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Compartmental responses of the respiratory tract to *Staphylococcus aureus*

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Abbreviation List

ACME	Arginine catabolic mobile element
ALP	Alkaline phosphatase
AP1	Activator protein 1
AQP3	Aquaporin3
ATF3	Activating transcription factor3
BALT	Bronchial associated lymphoid tissue
BEGM	Bronchial epithelial growth medium
BHI	Brain heart infusion
C/EBP	CCAAT-enhancer binding protein
CALM	Confocal and advanced light microscopy
CA-MRSA	Community associated MRSA
CD14	Cluster of differentiation 14
cDNA	Complementary DNA
CHIPS	Chemotaxis inhibitory protein of staphylococci
CID	Centre for Infectious Diseases
CK	Cytokeratin
ClfB	Clumping factor B
CpG	Deoxycytidyl-deoxyguanosine
CUE	Coupling of ubiquitin binding to endoplasmic reticulum domain
CYLD	Cylindromatosis
DAPI	4’,6-diamidino-2-phenylindole
DAXX	Death domain interacting protein
DD	Death domain
DMEM	Dulbecco’s modified eagle’s medium
DMSO	Dymethyl sulphoxide
dsRNA	Double stranded RNA
EDTA	Ethylene diamine tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
EPCAM	Epithelial cell adhesion molecule
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GAT	GGA and Tom1 domain
GI	Gastrointestinal
HAECs	Human airway epithelial cells
HBSS	Hanks balanced salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
HRP	Horseradish peroxidase
HSV-1	Herpes Simplex Virus-1
HV	Healthy volunteer
IFN	Interferon
IKK	IκB kinase
IL-1	Interleukin-1
IL-10	Interleukin-10
IL-12	Interleukin-12
IL-1RAcP	IL-1R-associated protein

IL-1RI	Type I interleukin-1 receptor
IL-6	Interleukin-6
IL-8	Interleukin-8
IRAK	IL-1 receptor associated kinase
IRF3	Interferon regulatory factor 3
IκB	Inhibitor kappaB
JNK	c-Jun N-terminal kinase
LBP	Lipopolysaccharide-binding protein
LPS	Lipopolysaccharide
LRRCT	Leucine rich repeat C terminal domain
LRRs	Leucine rich repeats
LTA	Lipoteichoic acid
Mal	MyD88 adaptor-like protein
MAPK	Mitogen activated protein kinases
MCP-1	Monocyte chemotactic protein-1
MEM	Minimum essential medium eagle
MFP	Methionine-phenylalanine-proline
MIP-1	Macrophage inflammatory protein-1
m-RNA	Messenger RNA
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSCRAMMs	Microbial surface components recognizing adhesive matrix molecules
MyD88	Myeloid differentiation protein 88
NF-κB	Nuclear factor-kappa B
NK	Natural killer cell
NOD	Nucleotide-binding oligomerization domain
NSCLC	Non-small cell lung cancer
ODN	Oligodeoxynucleotide
PAMPs	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PBP	Penicillin binding protein
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pDCs	Plasmacytoid dendritic cells
PGN	Peptidoglycan
PPARγ	Peroxisome proliferator activated receptor gamma
PtdIns(3)P	Phosphatidylinositol-3'-phosphate
PtdIns(3,4,5)P	Phosphatidylinositol-3,4,5-phosphate
PVL	Panton-Valentine leukocidin
QMRI	Queen's Medical Research Institute
QPCR	Quantitative PCR
RANTES	Regulated upon activation normal T cell expressed and secrete
RIG-I	Retinoic acid inducible gene-I
RLRs	(RIG-I)- like receptors
RT-PCR	Reverse transcriptase - PCR
SasG	*S. aureus* surface protein G
SLPI	Secretory leukocyte protease inhibitor

SNPs	Single nucleotide polymorphisms
SOCS-1	Suppressor of cytokine signaling-1
SP-C	Surfactant protein-C
ssRNA	Single stranded RNA
TAB2	TAK1 binding protein 2
TAK1	Transforming growth factor-activated protein kinase 1
TBD	Tom1 binding domain
TGF-β	Transforming growth factor-β
TIR	Toll/Interleukin-1 receptor homology domain
TIRAP	TIR associated protein
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-alpha
Tollip	Toll-interacting protein
TRAF6	TNF-receptor associated factor-6
TRAM	TRIF-related adaptor molecule
TRIF	TIR domain containing adaptor inducing IFN-β
TSB	Tryptic soya broth
TSST-1	Toxic shock syndrome toxin-1
VAP	Ventilator associated pneumonia

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Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) is an important nosocomial pathogen associated with significant morbidity and mortality. Previous colonisation with this pathogen is a risk factor for the development of subsequent infection. Toll-like receptors (TLRs) are a family of transmembrane receptors of the innate immune system that recognize pathogen-associated molecular patterns. The role of nasal colonisation of *S. aureus* has started to receive more attention. In spite of this, there are not enough studies looking at its effects on human primary nasal epithelial cells and their response to TLR ligands. The respiratory tract itself seems to pose a contradiction given by the clinical observation that its upper portion (nasal compartment) allows the growth of bacteria, acting like a reservoir, whereas the lower portion (lung compartment) reacts with an exuberant inflammatory response to the same organisms, as noted during pneumonia. The mechanism related with this phenomenon remains to be elucidated. A negative regulator of the TLR signalling cascade called toll-interacting protein (tollip) has been demonstrated to induce hypo-responsiveness in the gastrointestinal tract in the presence of bacteria. So far, tollip has not been demonstrated in the respiratory tract.

Aims: To compare the responses of the upper and lower respiratory tract to TLR ligands, to characterise the role of tollip in the respiratory tract and its effects in the induction of tolerance, and to determine the cellular response to nasal carriage of *S. aureus*.

Materials and Methods: The cell line RPMI 2650 (representative of nasal epithelium) and the cell line A549 (representative of type II alveolar epithelium) were used to establish the cytokine response to stimulation with TLR ligands and to demonstrate the presence of tollip protein by immunocytochemistry and enzyme-linked immunosorbent assay (ELISA). Primary human nasal epithelial and type II alveolar epithelial cells were isolated and cultured from consented subjects. The cytokine response to stimulation was measured using cytokine bead array and the presence of tollip was determined by immunofluorescence and quantitative polymerase chain reaction. The presence of TLRs was assessed by immunocytochemistry in primary nasal and type II alveolar epithelial cells and the response to stimulation with the TLR9 agonist CpG-C ODN was assessed in these cells as well as in primary human type II alveolar epithelial cells. Subjects were also assessed for nasal carriage of *S. aureus* and their associated cytokine responses.

Results: The RPMI 2650 cell line, despite retaining phenotypic characteristics of the nasal epithelium, appears unresponsive to stimulation with TLR ligands. In contrast, the A549 cell line responded significantly to stimulation with TLR ligands. Primary human nasal epithelial cells responded by secreting higher amounts of interleukin (IL)-8 and IL-6 in response to stimulation with *S. aureus* peptidoglycan (PGN) and tumour necrosis factor alpha (TNF-α) with a strong trend toward statistical significance. These cells did not respond to stimulation with *Pseudomonas aeruginosa* LPS. Primary type II alveolar epithelial cells responded significantly to stimulation with *S. aureus* PGN by increasing the secretion of IL-8, IL-6, IL-1β, TNF-α and IL-10 into cultured supernatant. Cells from the upper respiratory tract

displayed a more tolerant phenotype given by the lower levels in cytokine production in response to stimulation with *S. aureus* PGN, in contrast to alveolar epithelial cells. TLRs were identified in primary nasal epithelial cells. The negative regulator tollip was identified in cell lines as well as primary cells of the respiratory tract in its three segments: nasal, bronchial and type II alveolar. It was not possible to demonstrate an up-regulation of tollip after stimulation with TLR ligands in any of the cell types studied, although, it was possible to observe a significantly higher constitutive level in tollip mRNA transcripts from primary nasal epithelial cells in comparison to type II alveolar epithelial cells. TLR9 was identified in human primary nasal epithelial cells, although it was not possible to observe an increase in cytokine production after stimulation with a TLR9 agonist. TLR9 was expressed strongly in primary type II alveolar epithelial cells which responded by significantly increasing IL-8 production after stimulation with CpG-C ODN.

Primary nasal epithelial cells from individuals who carry *S. aureus* exhibit a pro-inflammatory profile, as evidenced by higher basal levels of IL-8 and IL-6 in comparison to non-colonised controls.

Conclusion: The upper respiratory tract epithelium displays a tolerant phenotype in response to stimulation with TLR ligands in comparison to the lower respiratory epithelium, potentially favouring nasal colonisation by *S. aureus*. Tollip m-RNA transcripts appear to be up-regulated constitutively in the nasal epithelium which might favour this response. *Staphylococcus aureus* colonisation is however associated with a local pro-inflammatory state in the nasal epithelium of carrier individuals.

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Declaration

All the experiments and procedures presented in this thesis were performed by the author unless otherwise indicated in the text.

CHAPTER ONE

INTRODUCTION

1 Introduction

1.1 Innate Immunity

The interaction between bacteria and humans has proved to be an enigma, whereas their coexistence has been demonstrated by the fact that the mitochondria is a remnant of bacteria illustrating the nature of the symbiotic relationship during evolution (Andersson, S. et al, 2003), the fact that the same bacteria that inhabit humans can also provide a lethal challenge for their immune system have not only produced as a result a very complex immune system but also fuelled a great deal of research in both fields: microbiology and immunology.

The innate immune system confers the first line of defence against the environment in a host multicellular organism. It can be considered as a collection of distinct subsystems or modules that carry out different functions in host defence (Medzhitov, R., 2007). These modules consist of mechanical barriers or flushing actions (e.g. skin or mucosal epithelium, peristalsis, cilia, fluid flow in urinary tract and GI tract), chemicals (gastric acid, sweat, sebaceous secretions and bile), production of circulating or mucosal proteins (e.g. acute phase proteins, complement, and interferons) and finally, circulating effector cells such as phagocytes, natural killer cells, eosinophils, basophils, and mast cells. The final desired outcome is the detection, neutralization and killing of invading pathogens, restoring homeostasis and allowing the host to survive. Evolution has helped multicellular organisms to refine and adapt their immune systems in order to

survive. Much initial immunological research was directed toward the fascination with the development of antibody responses to infections and the development of vaccination due to its strong protective effect by the development of immunological memory (Morgan, A. and Parker, S., 2007). However, further observation of these responses allowed the realization of the limitations that the adaptive immune response itself has including the relatively slow generation of T and B cell receptors during the development of these cells and, the need for them to be “reinvented” by every generation of lymphocytes (Janeway, C., 1992). Furthermore, the time-line needed to achieve such a precise response is around 3-5 days, by which, if there was not another “more efficient” line of defence, the host would certainly succumb to the deleterious effects of the invading pathogen (Medzhitov, R. and Janeway, C., 2000). However 1988 heralded a new era in the study of immunity, when the first receptor of the Toll family was identified in *Drosophila melanogaster* by Hashimoto, C., et al, 1988 as a key component of a signalling pathway controlling dorso-ventral polarity in fly embryos. It was noted that it encoded a transmembrane protein with a large extracellular domain containing leucine rich repeats (LRRs). Further studies by Gay, N. and Keith, F., 1991 demonstrated that the sequence of the cytoplasmic domain of the Toll proteins was remarkably similar to the cytoplasmic domain of the mammalian interleukin-1 receptor. It became apparent later that, the activation of the interleukin-1 receptor in humans and Toll in Drosophila generates a cascade of events that finally leads to the activation of the transcription factors of the nuclear factor-κB (NF-κB) family. Subsequent linkage to the immune role of Toll was

demonstrated in studies by Hoffmann's group (Lemaitre, B., et al, 1996) when it was observed that Drosophila with a loss of function mutation in the Toll gene were highly susceptible to fungal infection, although this mutation did not impair the response to bacterial infections. We now know that Drosophila has in total eight Toll-like proteins and that the other members of the family are programmed to recognize bacterial pathogens and induce antibacterial responses (Imler, J. and Hoffmann, J., 2000). It was not until 1997 that human homologues of Toll were identified by Janeway's group and were called Toll-like receptors (TLRs) (Medzhitov, R., et al, 1997). The first characterized receptor was Toll-like receptor 4 (TLR4) which was shown to induce activation of the NF-κB pathway, and by doing so, induce the expression of cytokines and co-stimulatory molecules that are necessary for adaptive immune responses. Following the discovery of Toll-like receptor 2 (TLR2), it became apparent that mice with a mutation in this protein had a normal response to LPS but were resistant to peptidoglycan and lipoproteins, which are key components of the cell wall of Gram-positive bacteria (Takeuchi, O., et al, 1999; Takeuchi, O., et al, 2000). The study by Wright and others, described CD14 as one of the accessory proteins required for the interaction of LPS with TLR4 and lipopolysaccharide-binding protein (LBP) (Wright, S., et al, 1990).

Soon came the discovery that double-stranded viral RNA was recognised specifically by TLR3 (Alexopoulou, L., et al, 2001) and that unmethylated bacterial DNA is recognized by TLR9 (Hemmi, H., et al, 2000), these allowed for

diversity of the system to be recognized as well as the role of the interplay in its member proteins. Data also began to emerge suggesting collaboration among different receptors in order to recognise and mount an inflammatory response against invading pathogens. Further research provided evidence that TLR1, TLR2 and TLR6 form an heterodimer to aid the recognition of Gram-positive bacteria (Ozinsky, A., et al, 2000) as well as a wider range of pathogens such as *Borrelia burgdorferi, Treponema pallidum* and *Mycoplasma fermentans* (Khor, C., et al, 2007). More specifically, tri-acylated bacterial lipoproteins are recognized by TLR1/TLR2 (Takeuchi, O., et al, 2002), whereas diacylated lipopeptides and zymosan (a component of the fungal cell wall) are recognized by TLR2/TLR6 (Kataoka, K., et al, 2002 and Underhill, D., et al, 1999). Other researchers such as Sugawara, I., et al, in 2003 have linked the TLR2 response to *Mycobacterium tuberculosis* and West, A., et al, 2006 to *Neisseria* spp and *Trypanosoma cruzi*. TLR5 recognizes flagellin from Gram-negative bacteria (Zhang, Z., et al 2005), and TLR7 and 8 recognise single stranded RNA (Mogensen, T. and Paludan, S., 2005). For a summary of the distinct TLR and ligands please refer to table 1.1.

Receptor	Localization	Ligand	Microorganism
TLR1/2	Cell surface	Triacyl lipopeptides	Bacteria
TLR2/6	Cell surface	Diacyl lipopeptides	*Mycoplasma*
		Lipoteichoic acid	Gram positive bacteria
TLR2	Cell surface	Lipoproteins	Bacteria
		Peptidoglycan	Gram positive and negative bacteria
		Lipoarabinomannan	Mycobacteria
		Porins	*Neisseria*
		GPI-mucin	Protozoa
		Zymosan	Fungi
		B-Glycan	Fungi
TLR3	Cell surface / endosomes	dsRNA	Viruses
TLR4	Cell surface	LPS	Gram-negative bacteria
TLR5	Cell surface	Flagellin	Flagellated bacteria
TLR7/8	Endosome	ssRNA	RNA viruses
TLR9	Endosome	CpG DNA	Bacteria
			Viruses

Table 1.1 Toll like receptors and ligands recognised by the innate immune system. dsRNA: double stranded RNA, ssRNA: single stranded RNA. Modified from Mogensen, T., 2009.

A much clearer picture of how the immune system as a whole functions has now emerged. The innate arm provides physical and chemical barriers to infection, effector cells able to act in a short period of time and circulatory or mucosal proteins which serve as amplifiers and perform the fine tuning of the response.

Environmental pressure has allowed for a relatively limited number of receptors able to recognize evolutionarily conserved structures of pathogens called pathogen-associated molecular pattern (PAMPs) which have in common the fact that they are invariant among entire classes of pathogens, essential for survival and distinguishable from the host's own patterns (Medzhitov, R., 2007). These characteristics allow an early and timely recognition of dangerous pathogens, in order that they be neutralised and killed.

On the other hand, the adaptive immune arm provides elimination of pathogens in the late phase of infection and the generation of immunological memory; this is achieved by clonal rearrangements from a broad repertoire of antigen-specific receptors on lymphocytes which provide specificity. These two systems work together orchestrating early pathogen recognition with the beneficial long term effects of immunological memory. This was particularly noted after the observation by Unanue, E., in 1997 that although innate immunity was crucial for controlling an infection, it is insufficient in order to achieve pathogen clearance. Although this might be true for symptomatic infections, in contrast, asymptomatic infections pose a challenge since little is known about the physiological aspects of incomplete clearance of the colonising organism by innate host-defence mechanisms.

Other more recently discovered components of the innate immune system include retinoic acid-inducible gene I (RIG-I)–like receptors (RLRs), and

nucleotide-binding oligomerization domain (NOD)-like receptors among others, but their mechanisms of action are out of the scope of the current discussion. For further information about these please refer to the review by Mogensen, T., 2009. Since the main focus of this research is in the innate immune response to bacterial pathogens, I will focus further on the TLRs that are primarily involved in this part of the immune response.

1.1.1 TLR2 signalling

The original clue about the role of TLR2 in the host response to staphylococcal infection was given when TLR2 knockout mice were noted to be highly susceptible to *S. aureus* (Takeuchi, O., et al, 2000). During a model of intranasal infection, Gonzalez-Zorn, B., et al, 2005 noted that TLR2 deficient mice had a 10-fold higher nasal carriage of *S. aureus* when compared to wild type mice.

Human TLR2 is a type I transmembrane protein composed of an intracellular (Toll/Interleukin-1 receptor homology domain,-TIR domain), a transmembrane and extracellular domains, in which the N-terminal domain is represented in the extracellular portion. It is different from the other TLRs because requires heterotypic interaction with TLR1 and TLR6 which accounts for the recognition of a wide range of PAMPs (Ozinksky, A., et al, 2000 and Takeuchi, O., et al, 2002).

When Jin, M., et al, 2007 resolved the crystal structure of TLR2 and TLR1, they showed that, the high content of leucine rich repeats (LRR) gives the receptor its concave surfaces and that TLR1 and TLR2 were formed as two parts of a m-shape complex with the lipopeptide Pam_3CSK4 in the middle binding the two receptor proteins together, the two N-terminal domains extending outwards in opposite directions and the LRRCT molecules converging at the centre (Jin, M., et al, 2007 and Jin, M., and Lee, J., 2008). The TLR1/TLR2 dimer recognizes triacylated lipopeptide (Miyake, K., 2007). Triantafillou, M., et al, 2006 showed using fluorescence techniques that the TLR2/TLR1 heterodimers and TLR2/TLR6 pre-exist and internalize with the ligand as their formation is not induced by agonists to the complex or PAMPs. The situation differs with TLR2/6, as they recognise diacylated lipopeptide and there are important changes in the dimerization interface that promotes heterodimerization, and the interaction of the complex with the lipid scavenger receptor molecule CD36 is not preformed but induced by the ligand (Triantafilou, M., et al, 2006), they also respond to stimulation with zymosan, a component of the fungal cell wall (Underhill, D., et al, 1999; Zahringer, U., et al, 2008; Miyake, K., 2007 and Kataoka, K., et al, 2002).

Activation by the ligand promotes dimerization of the receptor and the TIR domains of TLR2 and MyD88 bind. The presence of the adaptor protein MyD88 (myeloid differentiation protein) and TIRAP/Mal (TIR associated protein and MyD88-adaptor like protein) are crucial for the transduction of the signal since

Takeuchi, O., et al in 2000, noted that mice deficient in MyD88 were more susceptible to systemic infection with *S. aureus* in comparison to wild type mice (Figure 1.1). Further explanation of the responses associated with the MyD88 activation pathway will be covered in section 1.2.

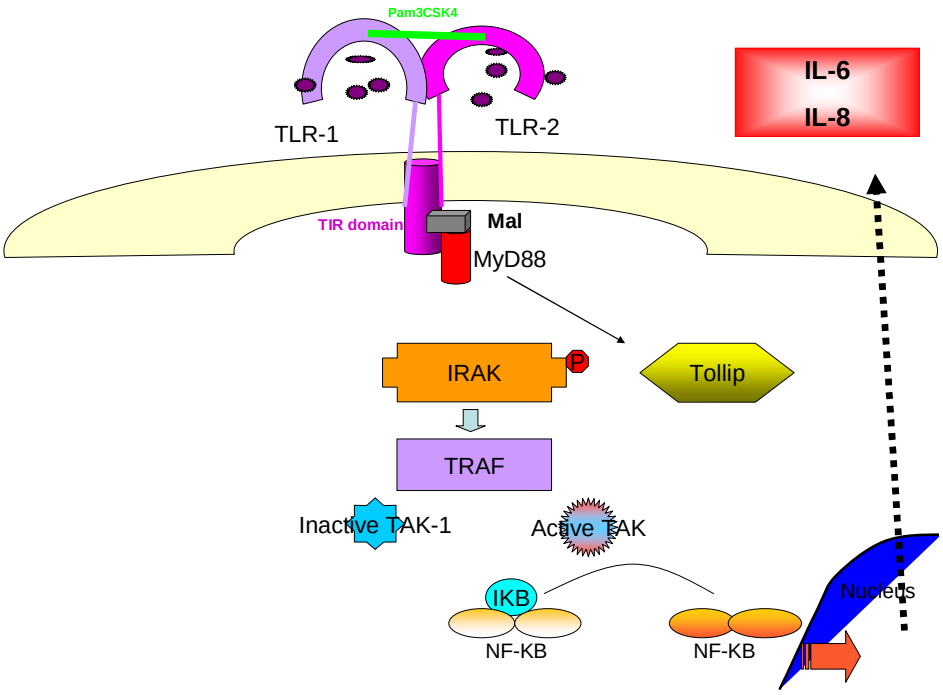

Figure 1.1 Mechanism of TLR-2/1 signalling.
Interaction of *S. aureus* with TLR2/1 heterodimer is followed by binding of the TIR portion of the receptor with MyD88, which subsequently recruits IRAK and associates with TRAF. TRAF ubiquitinates itself and activates TAK1. TAK1 mediates the activation of IKK complex which results in the phosphorylation of the inhibitor IκB protein which is followed by the translocation to the nucleus and activation of NF-κB, inducing the release of pro-inflammatory cytokines IL-8 and IL-6.
TIR domain, Toll/Interleukin-1 receptor homology domain; IRAK, IL-1 Receptor associated kinase; Tollip, toll-interacting protein; TRAF6, TBF-receptor associated factor 6; TAK1, transforming growth factor-activated protein kinase 1; IKK, IκB kinase.

Infection with *S. aureus* is also highly associated with sepsis and septic shock (Ammerlaan, H., et al, 2009 and Artero, A., et al 2010). This response happens when some of the main components of its outer membrane enter in contact with cells of the innate immune system. Under these circumstances and particularly due to the actions of TLR2, the peptidoglycan (PGN) and lipoteichoic acid (LTA) from this bacterium activate a pro-inflammatory cascade which is then associated with the release of pro-inflammatory cytokines into the bloodstream such as IL-1 and TNF-α which cause the increase in temperature (fever), recruitment of white blood cells (leukocytosis), increase of cardiac rate and respiratory rate that give the clinical presentation of sepsis.

1.1.2 TLR4 signalling

Janeway proposed LPS as the key molecule being recognised from pathogenic microorganisms due to its potent stimulatory properties in immune cells (Janeway, C., 1989). TLR4 was the first characterized receptor shown to induce activation of the NF-κB pathway, in response to LPS, this discovery was followed by Schumann's and coworkers in 1990 when they noted that a serum protein they called LPS binding protein (LBP) had the ability to solubilise LPS from the bacterium (Schumann, R., et al, 1990) and transfer it onto the leukocyte external membrane protein CD14 (Wright, S., et al, 1990). CD14 increases the sensitivity of leukocytes to LPS and reduces the binding affinity to picomolar concentrations. This allows transfer of LPS to the accessory molecule MD-2

which is associated with the extracellular domain of TLR4 (Gioannini, T., et al, 2004 and Gruber, A., et al, 2004). Recent studies have established that the TRL4 extracellular domain forms a rigid curved solenoid-like structure with MD-2 bound at two conserved sites in the amino-terminal region of TLR4 (Kim, H., et al, 2007 and Ohto, U., et al, 2007). The binding of the LPS ligand is followed by conformational changes in the receptor and homodimerization of the two cytoplasmic Toll interleukin-1 receptor domains (TIR) (Bryant, C., et al, 2010 and Saitoh, S., et al, 2004). The association of the TIR domains provides a scaffold for the recruitment of adaptor proteins to form an active signalling complex. The TLR4 pathway is particularly complex and requires the involvement for four different adaptors: MyD88, Mal, TIR domain-containing adaptor inducing IFN-β (TRIF) and TRIF-related adaptor molecule (TRAM) (Mogensen, T., 2009). The final outcome is two major signalling pathways, one involving MyD88, which induces the activation of NF-κB and mitogen-activated protein kinases (MAPK), as well as the activation of the transcription factor activator protein1 (AP1) (Chang, L., and Karin, M., 2001), these control the induction of proinflammatory cytokines. The second pathway is independent from MyD88 but dependent on TRIF, this event activates the transcription factor interferon regulatory factor 3 (IRF3), which then promotes the transcription of Interferonβ and the chemokine regulated upon activation normal T cell expressed and secrete (RANTES), as well as of Interferon inducible genes (Bryant, C., et al, 2010). This pathway also appears to mediate a late phase NF-

κB activation which potentiates the on-going inflammatory response that the MyD88 pathway already started (Figure 1.2).

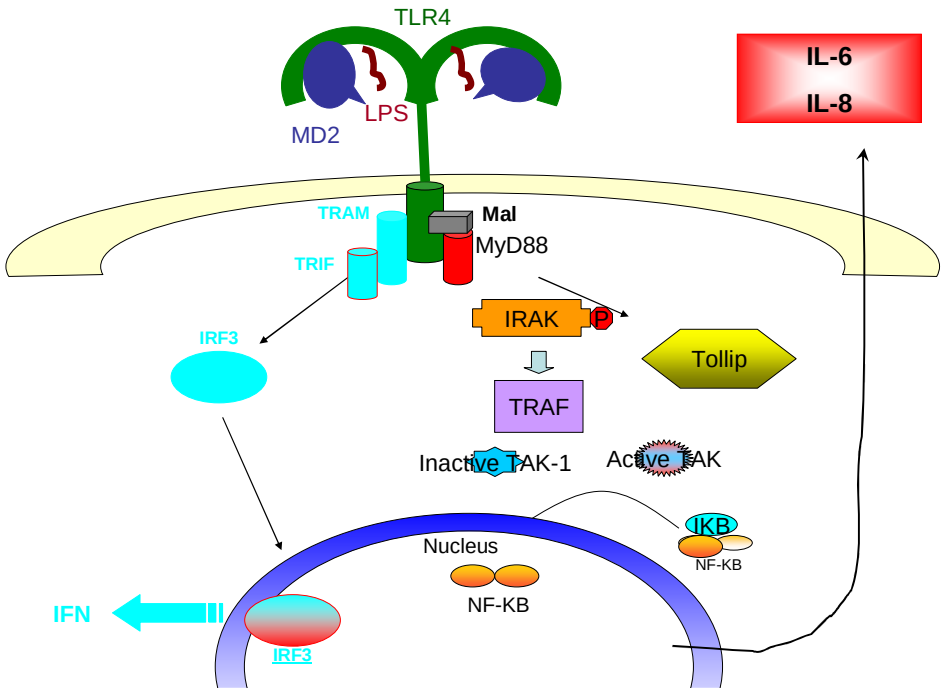

Figure 1.2 Mechanism of TLR-4 signalling.
Interaction with LPS is followed by binding of the TIR portion of the receptor with MyD88, which signals as described earlier and is associated with the production of proinflammatory cytokines. The TRAM dependent pathway stimulates the activation of IRF3 which induces interferon production and interferon associated genes
IRAK, IL-1 Receptor associated kinase; Tollip, toll-interacting protein; TRAF6, TBF-receptor associated factor 6; TAK1, transforming growth factor-activated protein kinase 1; IKK, IκB kinase; TRAM, TRIF-related adaptor molecule; TRIF, TIR domain-containing adaptor inducing IFN-β; IRF3, interferon regulatory factor 3; IFN, interferon.

1.1.3 TLR9 signalling

The original observation of the role of bacterial DNA on the inflammatory response was performed in 1984 by Tokunaga, T. and coworkers, when they observed that bacterial DNA isolated from *Mycobacterium bovis* strain BCG had strong antitumor effects given by the regression and prevention of metastasis of a carcinoma in CDF-1 mice and line 10 tumor of strain 2 guinea pigs. They went further and synthesized oligonucleotides derived from the same strain and observed that specific oligonucleotide sequences were associated in inducing the activation of natural killer cells (NK) and also stimulated mouse spleen cells to produce interferon (IFN) alpha and gamma (Tokunaga, T., et al, 1992). These observations triggered a great interest in the potential use of bacterial DNA synthetic oligonucleotides as potential antitumour agents. Further studies demonstrated in detail the stimulatory effects that bacterial DNA has on mammalian immune cells and attributed these activities to the presence of unmethylated CpG dinucleotides which are highly abundant in prokaryotic DNA. Finally by 2000, Akira's group published the sequence of TLR9 (Hemmi, H., et al, 2000). In their study they created $TLR9^{-/-}$ mice which were completely unable to respond to stimulation with CpG DNA, but yet retained the ability to increase cytokine production in response to LPS challenge. Whilst wild type mice died after a lethal injection of CpG DNA, the TLR9 deficient mice not only showed resistance, but also failed to increase serum pro-inflammatory cytokine levels, in addition it was also noted that the CpG DNA induced T_H1 response was

abolished. They also determined that TLR9 signals through the use of the adaptor molecule MyD88. Using wild type mouse macrophages they observed that stimulation with CpG DNA increased the DNA binding activity of NF-κB, in contrast, mutant mice showed no increase in NF-κB activity after CpG DNA stimulation, whilst both strain's macrophages responded to LPS stimulation. Furthermore, when using an *in vitro* kinase assay, it was observed that wild type macrophages activated c-Jun N-terminal kinase (JNK) and IRAK in response to stimulation with CpG DNA, whilst activation of both kinases was completely abolished in TLR9 null macrophages.

Establishing the actual localization of the receptor proved challenging but the observation that inhibitors of endosomal maturation such as bafilomaycin A or chloroquine abolish CpG mediated cell activation, suggests that endosomal maturation precedes cell activation (Hemmi, H., et al, 2000). It appears that the intracellular localization of the receptor acts a self protection mechanism as other receptors implicated in the recognition of DNA such as TRL7 and TLR8 are also located intracellularly as a manner to avoid auto-immunity (Christensen, S., et al, 2005 and Christensen, S., et al, 2006). The exact localization of the receptor and the kinetics involved with it, still remain to be defined since multiple reports colocalize it within the early and late endosomes, lysosomes and the endoplasmic reticulum (Barton, G. and Kagan, K., 2009).

The TLR9 pathway appears to behave similarly to the other TLRs in that it also signals through a MyD88 dependent route as demonstrated by the observation that dominant negative versions of MyD88, IRAK and TRAF inhibit CpG mediated cellular activation, furthermore, mutant mice lacking TLR9 or MyD88 fail to mount an immune response after exposure to CpG (Hacker, H., et al, 2000). The final outcome is the activation of the transcription factor NF-κB with the associated production of pro-inflammatory cytokines (Figure 1.3)

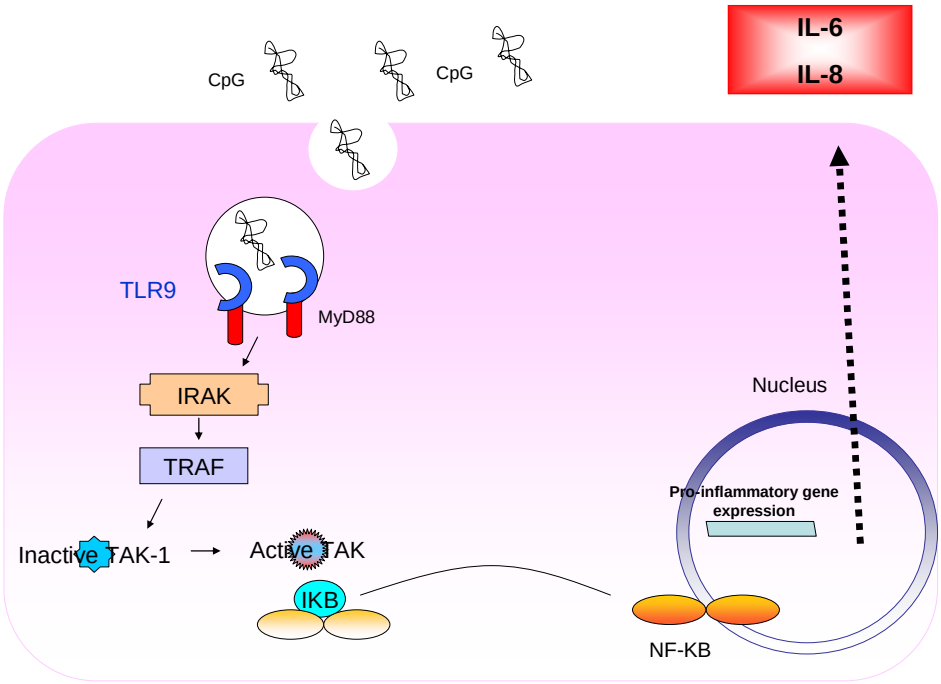

Figure 1.3 Mechanism of TLR9 signalling
CpG oligonucleotides are internalised through endocytosis, TLR9 is located within the endosomal vesicles and this transduces an intracytoplasmic activation signal dependent on MyD88.

1.2 Mechanism of action of the TLR pathway

Once the TLR has engaged its ligand, it starts triggering a cascade of events using different adaptors which modulate the specificity of the response. The pathways can be classified according to the use of specific adaptors (dependent or independent of MyD88 or TRIF and also on the activation of different transcription factors (NF-κB, mitogen-activated protein kinases (MAPKs) and interferon regulatory factors (IRFs) (Medzhitov, R., 2007 and Mogensen, T., 2009).

Numerous studies by Hemmi, H., et al, 2002, Horng, T., et al, 2002 and Yamamoto, M., et al, 2002, Yamamoto, M., et al, 2003 and Yamamoto, M., et al, 2003 inferred that MyD88 is the main adaptor molecule used by most TLRs, except TLR3 (Akira, S. and Takeda, K., 2004). Specifically for TLR4, other adaptors such as Mal, TRIF and TRAM are involved in signal transduction. For the purposes of this thesis I will focus on the MyD88 dependent pathway.

In response to stimulation, MyD88 is associated with the cytoplasmic tail of the TLR and recruits members of the interleukin-1 receptor associated kinase (IRAK) family (Burns, K., et al, 1998). Subsequently, IRAK4 and IRAK1/2 are phosphorylated sequentially allowing the association with TNF-receptor-associated factor 6 (TRAF6) (Suzuki, N., et al, 2002). A key step follows when TAK-1 binding protein 2 (TAB2) and TAB3 are recruited to the ubiquitinated

TRAF6, bringing TAK1 into proximity to the signalling complex, allowing its activation. TAK1-mediated activation of the IκB kinase (IKK) complex results in site specific phosphorylation of the inhibitory protein IκB (Wang, C., et al, 2001). Once phosphorylated, IκB undergoes proteasomal degradation allowing activation and translocation of the transcription factor NF-κB to the nucleus, where it binds to κB sites present in promoters and enhancers of a range of pro-inflammatory genes, which start being transcribed. NF-κB plays a pivotal role in the induction of inflammation given its ability to induce pro-inflammatory cytokines such as IL-1, IL-6, tumour necrosis factor-alpha (TNF-α) and chemokines such as IL-8 and regulated upon activation, normal T cell expressed and secreted (RANTES) (Mogensen, T., 2009). The final result is the activation and recruitment of leukocytes to sites of inflammation, enhanced phagocytosis of microbes, and cellular lysis through complement or NK cell activation and enhanced antigen presentation.

The inflammatory cytokines are produced by many cell types, particularly by macrophages and mast cells, their roles include activation of the endothelium and leukocytes as well as the induction of the acute-phase response. In addition chemokines which have been demonstrated to be produced by several cell types control leuckocyte extravasation and chemotaxis towards affected tissues Medzhitov, R., 2008). For a list of the cytokines involved in innate immunity in which I will focus during this work please see Table 1.2.

Cytokine	Principal cell source	Cell target	Biological effects
TNF	Macrophages, T & B cells, NK cells	Endothelial cells	Activation, inflammation, coagulation
		Neutrophils	Activation
		Hypothalamus	Fever
		Liver	Synthesis acute phase proteins
		Muscle, fat	Catabolism
IL-1	Macrophages, endothelial cells, epithelial cells	Endothelial cells	Activation, inflammation, coagulation
		Hypothalamus	Fever
		Liver	Synthesis acute phase proteins
IL-8	Macrophages, endothelial cells, epithelial cells	Neutrophils	Chemotaxis, activation
		Leukocytes	Migration into tissues
		Monocytes	
IL-6	Macrophages, endothelial cells, T cells, epithelial cells	Liver	Synthesis acute phase proteins
		B cells	Proliferation of antibody producing cells
		Epithelial cells	
IL-12	Macrophages, dendritic cells	T cells	T_H1 differentiation
		NK cells	IFN-γ synthesis, increased cytolytic activity
		T cells	
IL-10	Macrophages, T cells (mainly T_H2), Treg cells	Macrophages, T & B cells	Inhibition of IL-12 production
		Dendritic cells	Expression of costimulators and class II MHC molecules

Table 1.2 Summary of some of the cytokines involved in the innate immune response.
Modified from Tedgui, A., and Mallat, Z., 2006.

As the main focus of this research is the innate immune response to bacterial pathogens in the respiratory system, I will focus on this system.

1.3 Innate immunity in the respiratory system

The respiratory system has been divided for its study in upper and lower tracts.

The upper tract includes the nose, nasopharynx and larynx; is lined by mucous membranes on a vascular bed with ciliated epithelium in the surface. The lower respiratory tract includes the trachea and bronchi, which are lined with ciliated epithelium up to the terminal bronchioles that branch further leading to clusters of

alveoli. The alveoli are composed of two specialized cell types: the flattened squamous type I epithelial cells, which constitute approximately 93% of the alveolar epithelial surface area and type II epithelial cells which are cuboidal in shape, produce surfactant and also have the ability to proliferate and reconstitute the type I cells after lung injury (Crapo, J., et al, 1982 and Crompton, G., et al, 1999).

Although these anatomical divisions are useful for its study, functionally and immunologically, the respiratory system can be divided into: conducting airways and lung parenchyma; due to the different cell populations residing as well as to the differing levels of exposure to inhaled antigens throughout (Holt, P., et al, 2008). The conducting airways are lined with ciliated cells and secretory goblet cells that produce IgA and allow the mucocilliary clearance of inhaled antigens. The mucosa contains dendritic cells, T cells, B cells and macrophages. The airway mucosal dendritic cells are specialized for surveillance but lack the ability to efficiently present antigen (Stumbles, P., et al, 1998) T cells are also found in relatively high numbers in both: within the mucosa and in the underlying lamina propia. Furthermore, the airway mucosa also contains sites known as bronchial-associated lymphoid tissue (BALT) which resemble the tonsillar tissue or Peyer's patches in that they are discrete lymphoid cell aggregates underlying specialised epithelium (Moyron-Quiroz, J., et al, 2004).

The upper conducting airways are chronically exposed to pathogenic bacteria as well as to non-pathogenic environmental antigens, in order to avoid a continuous state of immunological activation, a specific clone of T cells with positive markers for Forkhead box P3 (FOXP3)$^{+}$CD4^{+}CD25+ regulatory T (TReg) cells, have been demonstrated to play a key role in the protection against the inflammatory consequences of airway infections and in protection against the development of allergies (Chen, C., et al, 2006; Holt, P., et al, 2008)

Because of its proximity to the exterior, the upper respiratory tract provides an attractive route to deliver medications to the lung compartment, for the study of the local response to pharmacological compounds, several cell lines have been used for the study of the cellular behaviour of the upper respiratory tract (Schmidt, M., et al, 1998). The cell line RPMI 2650 derived from an anaplastic squamous cell carcinoma of the nasal septum has been described as a suitable model for study of nasal cell behaviour since it closely resembles the nasal epithelium with respect to karyotype and cytokeratin polypeptide expression pattern (Salib, R., et al, 2005 and Bai, S., et al, 2007). This cell line has been reported to produce the cytokine transforming growth factor-β (TGF-β) as an autocrine growth factor.

The identification of potentially pathogenic antigens is facilitated by TLRs. Several groups have established the presence of TLRs in nasal epithelial tissue and primary cells in relation to primary nasal pathology (Wang, J., et al, 2007; Lane, A., et al, 2006; VanderMeer, J., et al, 2004).

The lung parenchyma comprises the alveoli which are separated by thin walls of interstitium containing pulmonary capillaries located in close contact with the alveolar space. During homeostasis, the main leukocyte population is made of alveolar macrophages (more than 90%) and the remainder are dendritic cells and T cells (Holt, P., et al, 2008). Airway epithelial cells constitute the first line of defence, not only do they form a physical barrier, but actively secrete into the interstitial fluid molecules such as mucins, surfactant, complement and complement cleavage products as well as antimicrobial peptides such as defensins, secretory leukocyte protease inhibitor (SLPI), elafin among others (Holt, P., et al, 2008). The production of antimicrobial peptides has also being noted as respiratory secretions possess antimicrobial activity (Travis, S., et al, 1999). SLPI, has been noted to have antimicrobial activity against Gram-negative and Gram-positive bacteria which is dependent on its N-terminal domain (Hiemstra, P., et al, 1996).

In addition, the lung epithelium also modulates airway smooth muscle activity, amplifies the host response to microorganisms through the secretion of chemokines and cytokines among other functions (Mayer, A. et al, 2007 and Holt, P., et al, 2008).

For its study, several researchers have used in-vitro models with cell lines from the lower respiratory tract, the most commonly used model constitutes the A549 cell line, which was established in 1972 by Lieber and coworkers from a human

alverolar cell carcinoma (Lieber, M., et al, 1976). Burvall, K., et al, 2003 determined that A549 cells constitutively produced the cytokines IL-8 and IL-6. Stimulation with TLR ligands has been associated with an increase in the cytokine secretion by these cells. Further work (Droemann, D. et al, 2003) identified the presence of TLR2 in these cells and (Guillot, L. et al, 2004) provided the evidence for a LPS-TLR4 dependent signalling pathway arguing the in the respiratory tract this receptor is sequestered in the cytosol as a way to maintain homeostasis. Although cell lines have helped greatly to the understanding of basic cellular behaviour, they have limitations related particularly in the case of cancer derived cell lines with the fact that their precursors are far from normality and therefore establishing assumptions of normal physiological using these models is inappropriate.

The establishment of primary cell culture techniques for the study of human cells of the respiratory tract has proven challenging but it has been achieved by several groups (Dobbs, L., 1990; Witherden, I., et al, 2004 and Fuchs, S., et al, 2003).

1.4 Negative regulation of the TLR pathway

The innate immune system can act as a double edged sword: on one hand it is necessary to detect, neutralise and kill invading microorganisms by the induction of inflammatory responses. On the other, an excessive inflammatory state will be detrimental for the host. There are several lines of evidence that determined the existence of an extensive negative regulator mechanism of the inflammatory

cascade in which the TLRs are involved (Cario, E., and Podolsky, D., 2005; Liew, F., et al, 2005; Shibolet, O. and Podolsky, D., 2007). These can be classified by their site of action: 1) extracellular: soluble decoy TLRs (Iwami, K., et al, 2000; LeBouder, E., et al, 2003); 2) membrane: reduced expression of TLRs (Otte, J., et al, 2004 and Otte, J. and Podolsky, D., 2004); 3) intracellular: short spliced forms of MyD88(MyD88s) (Burns, K., et al, 2003; Janssens, S., et al, 2002), interleukin-1 receptor-associated kinase-M (IRAK-M) (Kobayashi, K., et al, 2002; Wesche, H., et al, 1999), suppressor of cytokine signalling (SOCS-1) (Kinjyo, I., et al, 2002; Nakagawa, R., et al, 2002), NOD2 (Watanabe, T., et al, 2004; Watanabe, T., et al, 2005), Toll-interacting protein (Tollip) (Burns, K., et al 2000; Cario E., and Podolsky, D., 2005), the ubiquitin modifying enzyme A20 (Boone, D., et al, 2004; Krikos, A., et al, 1992; Opipari, A., et al 1990) and 4) nuclear: inhibitors affecting transcriptional output such as peroxisome proliferators-activated receptor-γ (PPARγ) (Dubuquoy, L., et al, 2003; Dubuquoy, L., et al, 2006), activating transcription factor 3 (ATF3) (Gilchrist, M., et al, 2006) and the tumour suppressor cylindromatosis (CYLD) (Yoshida, H., et al, 2005).

The gastrointestinal tract is a good example of a system constantly being challenged by exogenous and endogenous bacterial products. Furthermore, its role in the induction of immunological tolerance has been established by the discovery by Melmed and colleagues in 2003 that human intestinal cells are unresponsive to TLR-2 bacterial ligands. There are two reasons for these findings: first, the fact that intestinal epithelial cells express low levels of TLR2 and TLR6 and second,

the fact that these cells display high expression levels of tollip. These findings were corroborated by Otte, J., et al, 2004 when they also noted that intestinal epithelial cells responded to a short-term stimulation with LPS or lipoteichoic acid (LTA) by displaying a pro-inflammatory phenotype, whereas prolonged incubation with the same ligands was associated with an hyporesponsive state of the cells with no reactivation after a second challenge with bacterial products (nevertheless, the cells remained responsive to stimulation with TNF). On closer examination of the hypo-responsive cells they also noted a decrease in TLR surface expression but more markedly an increase in tollip mRNA and protein expression which seems to be likely to contribute to the tolerant state of these cells.

1.5 Tollip (Toll-interacting protein)

Tollip could possibly be best described as an endocytic adaptor protein (Brissoni, B., et al, 2006). Endocytic adaptors are proteins with domains for protein-protein or protein-lipid interactions and sites that lack catalytic ability for inducible post-translational modifications. They act at the junction between signalling and endocytosis (Katoh, Y., et al, 2004; Maldonado-Baez, L., and Wendland, B., 2006) and have a modular design consisting of combinations of discretely folded domains and unstructured regions that contain multiple peptide-binding motifs and it is this property that allows them to simultaneously interact with numerous

partners in an apparently synchronized fashion. The interactions can be classified depending on the interacting partner as lipid-adaptor, cargo-sorting-signal adaptor and accessory protein-adaptor interactions. The regulation of these adaptors happens through two main mechanisms: phosphorylation and ubiquitination.

First identified in 2000 by Burns and coworkers, through a yeast-two hybrid screen using the IL-1R accessory protein, in mice, tollip is a small protein consisting of 274 amino acids (a.a.). Using database searches, they identified homologues of tollip in humans and the nematode *Caenorhabditis elegans* which share 97% and 41% sequence identity respectively. Tollip messenger RNA (mRNA) species have been detected in a variety of tissues in mice including heart, lung, brain and testis (Burns, K., et al, 2000).

In humans, the *Tollip* gene is located on chromosome 11p15 and it comprises 6 exons that can be transcribed in at least four different isoforms (Schimming, T., et al, 2007). The gene codes for the full length tollip, which is a 274 a.a. protein. It appears to be a highly alternatively spliced gene generating several coding and non-coding variants. Alternate transcription of TLR pathways is a mechanism for generating combinatorial expansion of possible signalling outcomes, including repression of signalling at multiple points in the cascade (Jaresova, I., et al, 2007). The study from Wells, 2006 performed a systematic analysis of the FANTOM3 mouse data set, they found that as well as the adaptor molecules, the kinases in the TLR pathway produced multiple variant protein products through alternate

splicing. Their data set found a novel 220 a.a. tollip isoform generated from an alternate terminating exon that lacks the amino-terminal CUE domain. They predict that this amino-terminal truncated variant could exacerbate inflammatory signalling through TLR and IRAK pathways (Wells, C., et al, 2006)

Lo and coworkers, identified four variants: the human canonical form *Tollip A* transcribed from six exons, translating into a 274 a.a. protein with 94% a.a. identity to mouse *Tollip a*; *Tollip B* transcribed from five exons, (skipping exon 2) translated into a 224 a.a. protein with no Tom1 binding domain; *Tollip C* also transcribed from five exons, (skipping exon 3) translated into a 213 amino acid protein that lacks the C2-like domain; and *Tollip D* initiated from an alternate exon 1, transcribed across six exons, translated into a protein product of 205 amino acids (Lo, Y., et al, 2009). Three Tollip variants are detected in human peripheral blood monocytes and the four variants were also detected across a range of tissues with a brain-enriched profile which shows abundant expression of *Tollip A* (2.5 times more abundant than *Tollip B* and *Tollip C*). Analysis of *Tollip* transcripts in human and mouse has demonstrated rapid diversification of exon usage in this locus. A high proportion of macrophages expressed the canonical variant A as well as the human-specific variant *Tollip B* and only a small subgroup of the human monocytes in the population expressed *Tollip C*. These findings reveal that expression of *Tollip* variants in different macrophages provides a mechanism for diversifying innate immune signalling. All of the human tollip protein isoforms studied by this group retained the C-terminal regions including

the coupling of ubiquitin conjugation to endoplasmic reticulum degradation (CUE) domain. (Lo, Y., et al, 2009)

1.5.1 Tollip protein structure

Three main functional domains have been described in tollip: the Tom1 binding domain (TBD), a C2-like domain in the N-terminal portion and a CUE domain at the C-terminal portion (Figure 1.4).

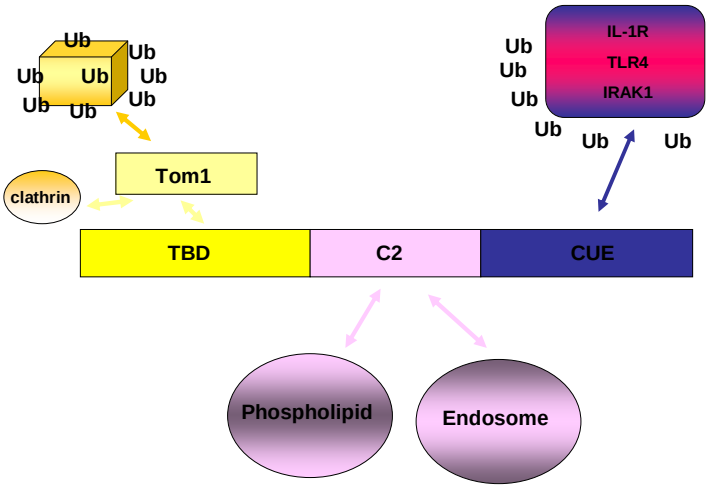

Figure 1.4 Tollip protein domains.
TBD: Tom1 binding domain, C2, C2 domain and CUE coupling of ubiquitin (Ub) binding to endoplasmic reticulum domain. Adapted from Lo, Y., et al, 2009.

1.5.1.1 Tom1 binding domain (TBD)

The TBD was identified by Yamakami and colleagues who also described Tom1 as a novel ubiquitin binding protein. It was noted that Tom1 interacts

with Tollip forming a high molecular mass complex both *in vivo* and *in vitro* (Yamakami, M., et al, 2003). They also found that the N-terminal portion is required for the interaction with the GGA and Tom1 domain (GAT) domain of Tom1. It has been observed that there are direct interactions of Tom1 and tollip with ubiquitin chains and that the ubiquitin binding capacity of Tom1 is not suppressed by the presence of tollip or vice versa suggesting that both Tom1 and tollip in the complex can bind with the ubiquitin chains simultaneously. They also propose a model in which a complex of Tom1 and tollip, (both of which are capable of binding with polyubiquitin chains) recruits polyubiquitinated proteins to clathrin, one of the main proteins involved in endocytosis (Yamakami, M., et al, 2003).

1.5.1.2 The C2 domain

The C2 domain, in the central and most conserved region, is responsible for association with endosomal membranes. The C2 domain is a calcium binding domain and by selectively binding phosphatidylinositol-3'-phosphate (PtdIns(3)P) and phosphatidylinositol-3,4,5-phosphate (PtdIns(3,4,5)P) which are downstream products of PI3 kinase, it has been shown to be involved in multiple signalling events and trafficking (Li, T., et al, 2004). Treatment with the PI3 kinase inhibitor wortmannin alleviated tollip mediated suppression of NF-κB. It was noted that the disruption of the C2-like domain of tollip disrupts the negative regulation that tollip exerts on LPS induced NF-κB activation,

implying that this domain plays a vital role in controlling TLR4 mediated signalling events (Li, T., et al, 2004).

1.5.1.3 The coupling of ubiquitin binding to endoplasmic reticulum (CUE) domain

The CUE domain is situated at the C-terminal end of tollip (Brissoni, B., et al, 2006) and is crucial for its interaction with the IL-1R. The CUE domain is also found in proteins involved in endosomal functions (Katoh, Y., et al, 2004).

The importance of this domain was observed by Brissoni and colleagues: they were aware that following stimulation with IL-1, the type I interleukin-1 receptor (IL-1RI) is rapidly internalised and conjugated by multiple monoubiquitin moieties. After they generated of a tollip mutant at the MFP (methionine-phenylalanine-proline) motif (residues 240-242) in the CUE domain (which is also a high affinity ubiquitin binding site), they observed that the mutation rendered tollip incapable of binding IL-1RI. They also showed that tollip is necessary for sorting of IL-1RI in late endosomes since it was noted in tollip deficient cells that significant levels of IL-1RI remained present in late endosomes after 3-6 hours post-stimulation. This accumulation of IL-1RI could be reversed by stable reconstitution of tollip-deficient cells with tollip but was not observed when empty vector was used, indicating that tollip and its capacity

to act as an ubiquitin receptor for IL-1RI is required for efficient sorting in late endosomes.

Tollip's CUE domain is also required for efficient degradation of IL-1RI. Tollip has specificity for endocytic cargo, which includes IL-1RI and other ubiquitinated TIR-family receptors (TLR2,4) with which it interacts through this domain (Zhang, G., and Ghosh, S., 2002). The regions around the CUE domain of tollip bind to unphosphorylated IRAK under resting conditions and also associate transiently with the TLRs and IL-1R upon stimulation. The same region can also be phosphorylated by IRAK. After activation, IRAK phosphorylates tollip, which leads to the dissociation of tollip from the complex. Exposing the CUE domain on tollip led to subsequent degradation by ubiquitination. The removal of tollip allows signalling to continue by freeing activated IRAK to bind to downstream TRAF6 (Zhang, G., and Ghosh, S., 2002).

1.5.2 Mechanism of action of tollip

Tollip seems to block ligand-independent Toll signalling. Immunoprecipitation experiments using HEK293T cells by Burns et al, showed that tollip is associated with type I interleukin-1 receptor (IL-1RI) and IL-1R-associated protein (IL-1RAcP) during the resting state. They also showed that tollip binds to IL-1 receptor associated kinase (IRAK) only in unstimulated cells, suggesting that Tollip-IRAK complexes are preformed in the cells.

Stimulation with IL-1β induces the aggregation of IL-1RI and IL-1RAcP, as well as the independent recruitment of MyD88. The binding of MyD88 and the tollip-IRAK complex, allows the death domains of MyD88 and IRAK to interact inducing progressive autophosphorylation of IRAK and causes dissociation of the complex. The inability to co-immunoprecipitate tollip and MyD88 suggests that they exist in distinct protein complexes, these findings are consistent with previous observations that both molecules are recruited independently to the IL-1R after stimulation (Burns, K., et al, 2000).

It has also been shown that the tollip-IRAK association takes place with the N-terminus side of IRAK and not with other death domain (DD) containing proteins (Burns, K., et al, 2000). The observation that tollip translocates to the membrane after stimulation with IL-1β, suggests that IRAK and tollip could be recruited in a complex and following IRAK phosphorylation, the complex may be disrupted. Furthermore, when tollip was co-expressed after IRAK over expression, IRAK activation was blocked suggesting that spontaneous activation of IRAK could be blocked once it is complexed with tollip (Burns, K., et al, 2000).

In addition to undergoing autophosphorylation, IRAK also phosphorylates tollip. Phosphorylation of tollip by activated IRAK may facilitate the dissociation of IRAK from tollip thereby allowing IRAK to escape the inhibitory effect of tollip

and subsequently modifying downstream signalling components (Burns, K., et al, 2000).

Studies by Li and coworkers using IRAK-deficient cells have shown that phosphorylation of IRAK is required for it to dissociate from the receptor and transduce signals to downstream molecules leading to NF-κB activation (Li, X., et al, 1999). Moreover, it is possible that phosphorylation of tollip may facilitate its dissociation from IRAK and subsequent degradation by ubiquitination as demonstrated by the fact that tollip contains a highly conserved CUE domain at the C terminus (230-270). As mentioned previously, similar domains in other proteins recruit ubiquitin conjugating enzymes, leading to proteosome dependent degradation (Zhang, G., and Ghosh, S., 2002).

Tollip has the ability to associate with TLR2 and TLR4. This was demonstrated by Bulut, Y., et al, 2001, when it was co-immunoprecipitated with TLR2 and TLR4 in human dermal and endothelial cells. Furthermore, Zhang et al showed that HA (haemagluttinin) tagged-tollip coprecipitates efficiently with TLR2 and TLR4 but it was significantly enhanced with the latter by the presence of MD-2, this suggests that structural changes in the cytoplasmic domain of TLR4 induced by MD2 are necessary for efficient binding to tollip (Zhang, G., and Ghosh, S., 2002). When Bulut, over-expressed tollip, they showed that it inhibits TLR2 and TLR4 mediated NF-κB activation in a dose-dependent manner in 293 T cells. This suggests that this molecule is also shared among the IL-1R, TLR4 and

TLR2 signalling pathways and that it limits proinflammatory signals from the innate immune system (Bulut, Y., et al, 2001). Moreover, in a study by Zhang, using dominant negative TLRs (truncated versions of TLRs that lack the intracellular signalling TIR domain) it was demonstrated that the sensing of *Pseudomonas aeruginosa* by human airway epithelial cells (HAECs) was specifically mediated by TLR5 (Zhang, Z., et al, 2005). After cloning tollip from a human cDNA library and over-expressing it in the same cells they found that significant blocking of TLR5 occurred. A dominant negative of TIR-domain containing adaptor protein (TIRAP) had no effect on *P. aeruginosa*-induced NF-κB activation. These results revealed a common initial part of the IL-1R signalling pathway involving MyD88, IRAK and its regulating protein tollip and TRAF6, which were utilized by TLR5 in response to *P. aeruginosa*.

So far, there is no evidence of tollip executing an inhibitor role in relation to the TLR9 activation pathway. The study from Yeo, S., et al, 2003 failed to demonstrate up-regulation of tollip after stimulation of RAW264.7 cells with CpG DNA, although IRAK was degraded and inhibited.

When Piao and colleagues studied primary human monocytes they observed that the over-expression of tollip successfully inhibited LPS-mediated activation of NF-κB and RANTES but did not affect TNF-induced NF-κB reporter activity (Piao, W., et al, 2009).

The generation of tollip knockout mice by Didierlaurent, provided valuable insight into how this protein interacts in the immune system. Tollip null mice exhibited normal IκB degradation and phosphorylation of MAPK by IL-1R, TLR2 and TLR4. There were no differences in the degradation of IRAK and the activation of dendritic cells and lymphocytes was not affected. However, it was noted that IL-6 and TNF-α production was decreased in tollip-deficient cells after stimulation with IL-1β or LPS and there were no differences in the production of the IFN-inducible genes *RANTES* or *ISG15* after poly (I-C) or LPS stimulation in tollip deficient mice. The above findings suggest that tollip controls the fine tuning of the inflammatory cytokine production in response to IL-1R and TLR pathways that are dependent on MyD88 signalling (Didierlaurent, A., et al, 2006).

Tollip is capable of self association or oligomerization to allow signals to be transduced efficiently (Zhang, G., and Ghosh, S., 2002). When they performed mutations in *tollip* they found that all *tollip* mutants associated efficiently with full length *tollip* except for the construct containing only the C2 domain (a.a.s 53-178). Therefore they concluded that self-association of tollip is mediated by both the N and C terminal regions of the protein and may result in formation of a stable protein complex.

More recent work by Ciarrocchi and colleagues has implicated tollip as a mediator of protein sumoylation (Ciarrocchi, A., et al, 2009). Sumoylation is a

post-translational modification of proteins that are involved in various cellular processes such as nuclear-cytosolic transport, transcriptional regulation, apoptosis, protein stability, response to stress and finally, progression through the cell cycle (Hay, R., 2005 and Zhao, J., 2007). Ciarrocchi found that tollip is sumoylated in more that one lysine residue by its association with the sumoylation enzyme SUMO-1 since they co-immunoprecipitated together. The most studied role of SUMO-1 modification on proteins is that of nuclear translocation. In cells that constitutively express the IL-1RI tollip deposited itself in small aggregates in the cytoplasm, in the perinuclear region in a position that may correspond to the Golgi apparatus, and/or to endosomes, and also in the nuclear bodies, where it also co-localises with SUMO-1 and the death domain interacting protein (DAXX) repressor. This finding suggests that tollip together with SUMO-1 are translocated into the nucleus and internalized into nuclear bodies (Ciarrocchi, A., et al, 2009).

1.5.3 Localisation of tollip:

Tollip appears to be a highly abundant intracellular protein. Brissoni and coworkers found it localised on early as well as in late endosomes and they think that is likely that the localisation of tollip in the different endosomal compartments is a dynamic and highly regulated process controlled in part through its internal C2 domain which is abundant on endocytic vesicles (Brissoni et al, 2006). This finding was also supported by the observation from Katoh

when they noted that HA tagged-tollip was localised on punctate structures distributed throughout the cytoplasm which partially overlapped with an early endosomal marker EEA-1; but not significantly with the cation-independent mannose-phosphate receptor, which tends to cycle between the trans-Golgi network and late endosomes, suggesting that tollip localises on part of the population of early endosomes (Katoh, Y., et al, 2004). Tollip mutants lacking the TBD, the CUE domain and both domains, all retained the ability to associate with endosomal structures. Therefore it is highly probable that the C2 domain determines endosomal localisation.

The fact that tollip has been recently implicated in sumoylation suggests a nuclear role for tollip as well as its involvement in nuclear cytoplasmic trafficking (Ciarrocchi, A., et al, 2009).

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1.5.4 Role of Tollip in the adaptive immune response

Tollip is not only a key player in the modulation of the innate immune response, but there is evidence that it also provides a link to adaptive immunity. This was observed by Ohnuma, K., et al, 2005 when using proteomic analysis they identified tollip and IRAK-1 as caveolin-1 interacting proteins in monocytes. Adenosine deaminase-binding protein (CD26) is a 110 kD type II transmembrane molecule which was originally characterised as a T cell activation antigen, preferentially expressed on a specific population of T

lymphocytes, particularly the subset of CD4+ memory T cells (Dang, N., et al, 1990 and Dang, N., et al, 1991). Ohnuma observed that after stimulation with CD26 tollip and IRAK1 dissociated from caveolin-1 and IRAK 1 was phosphorylated in the cytosol, leading to the upregulation of the costimulator for T lymphocyte activation molecule CD86 via the activation of NF-κB. It is likely that the result of this interaction is associated with T cell activation and proliferation of T memory cells.

The clinical demonstration of tollip's actions in the adaptive immune system was provided by Kimman and colleagues who found an association between interacting genes in the TLR signalling pathway and the antibody response to *Bordetella pertussis* vaccination. They collected data from 490 children for genotyping and single nucleotide polymorphisms (SNPs) selection and compared it to the levels of pertussis toxin (PT-IgG) titres. Using multifactor dimensionality reduction analysis they showed significant genetic interactions of SNPs in *tollip*, *TLR4* and *IRAK-1*. Children with wild type genotypes of both *tollip* and *TLR4* had a high chance of PT-IgG in the highest 33rd percentile. When an *IRAK-1* SNP was added to the combination (*tollip* and *TLR-4* SNPs) further increased the accuracy and was also significantly associated with high PT-IgG titres (Kimman, T., et al, 2008).

1.5.5 Role of Tollip in clinical medicine

Although not studied extensively in a clinical setting yet, some groups have started to look for the clinical relevance that tollip's multiple actions have. A study by Schimming, T., et al, 2007 found two polymorphisms in the *tollip* gene which appear to have an association with atopic dermatitis, a chronic inflammatory skin disease of multifactorial origin in which the evidence supports an inverse relationship between its development and the exposure to endotoxin (Kabesch, M., et al, 2003 and Kabesch, M., et al, 2004).

Hu demonstrated that tollip could be a negative regulator during the development of cardiac hypertrophy in C57BL/6 mice, as well as *in vitro* in neonatal cardiomyocytes, through the down regulation of the MyD88-dependent NF-κB activation pathway, suggesting a broader role of this protein in the myocardium (Hu, Y., et al, 2009).

Septic shock has been recognized as one of the main results of an uncontrolled inflammatory response, and there is evidence to support the concept that sepsis syndrome may be mediated by TLRs (Poltorak, A., et al, 1998). The group led by dib-Conquy could not demonstrate a difference between tollip m-RNA in sepsis patients and with healthy controls, however (dib-Conquy, M., et al, 2006).

1.6 *Staphylococcus aureus* epidemiology

Septic shock accounts for high morbidity and mortality, and the Gram-positive bacterium *Staphylococcus aureus* is one of the main species associated with this life-threatening disease (Artero, A., et al, 2010). The SEPIA study group (Ammerlaan, H., et al, 2009) found *S. aureus* bacteraemia to be significantly associated with 30-day mortality. Furthermore, the association between *S. aureus* and nosocomial infection, particularly ventilator-associated pneumonia VAP) has received increasing attention during the last decade (DeRyke, C., et al, 2005; Lynch, J., 2001 and Rello, J., et al, 1994). North American national surveillance programs have identified *S. aureus* as the second most common causative agent in hospital-acquired infections (Hidron A., et al, 2008).

Staphylococcus aureus is a Gram-positive bacterium. It is considered a very successful colonising agent and pathogen since it is very versatile and possesses multiple virulence factors that allow it to adhere, colonise and infect its host. It has been well known to cause epidemics (Chambers, H., 2005; Chambers, H. and DeLeo, F., 2009; Enright, M., et al, 2002; Robinson, D., et al, 2005 and Stewart, G. and Holt, R., 1963). It also has the ability to acquire resistance to antibiotics at intimidating speed: the first penicillin-resistant strains were reported in the mid 1940s and were associated with causes of infections in hospitalized patients (Barber, M., et al, 1949; Kirby, W., 1944). Very soon after, and extending up to the 1960s, the appearance of the penicillin resistant *S. aureus* strain phage type

80/81 was associated with hospital-acquired diseases as well as with infections in the community (Donahue, J. and Baldwin, J., 1966). After the introduction of methicillin in 1959, the phage type 80/81 strains disappeared and almost immediately were replaced by methicillin resistant *Staphylococcus aureus* (MRSA) strains which are now an increasing problem in current hospital medical practice (Chambers, H., 2001; Holt, R. and Stewart, G., 1963).

The prevalence of MRSA has been rising during the last two decades. In the United Kingdom, there was a sharp rise in the proportion of MRSA among all cases of *S. aureus* bacteraemia during the 1990s and reached its peak at 40-45% during 2001-2005. The implementation of strategies incorporating strict infection control measures was associated with a decrease to 36% in the incidence during 2007 has been seen. The initial increase was due to the successful spread of two epidemic MRSA strains EMRSA-15 (ST22-MRSA-IV) and EMRSA-16 (ST36-MRSA II) which were mostly linked to hospital settings (Enright, M., et al, 2002; Johnson, A., et al, 2005).

Simultaneously, in the early 2000s, the emergence was observed of a new MRSA strain which affected a completely different population of patients including nursery children, teams of contact sportsmen, prisoners, military recruits and homosexual men (Diederen, B. and Kluytmans, J., 2006 and Zetola, N., et al, 2005). This strain was called a community associated-MRSA strain (CA-MRSA) and its main characteristic of this emerging strain was the increased virulence due

to the production of a toxin able to cause pores in the membranes of white blood cells. This is called the Panton-Valentine leukocidin (PVL) (Vandenesch, F., et al, 2003).

The versatility of *S. aureus* to develop new antibiotic resistance was evidenced in Japan in 1997 at a time when severe MRSA infections were conventionally treated with glycopeptides (Hiramatsu, K., et al, 1997). Antibiotic resistance has been considered an established issue since 2002 when the first true vancomycin-resistant MRSA strain was identified from a diabetic foot ulcer which also had a vancomycin-resistant *Enterococcus faecalis* (Sievert, D., et al, 2008). The introduction of new antibiotics such as linezolid and daptomycin has not stopped the development of antibiotic resistance in MRSA. Whereas linezolid resistance has been reported during therapy (Meka, V., et al, 2004; Tsiodras, S., et al, 2001), daptomycin (a new lipopeptide) is not far ahead, and the development of resistance during treatment has again resulted in therapeutic failure (Hayden, M., et al, 2005; Mangili, A., et al, 2005; Marty, F., et al, 2006, Skiest, D., 2006; Vikram, H., et al, 2005). Therefore, we are now, more than ever, extremely vulnerable to this microorganism which has proven itself superior to all advances in modern medicine.

So, what makes these bacteria so special? There is evidence that multiple factors account for its success including the ability to adhere to host cells, to persist

intracellularly, to evade immune responses, to invade and penetrate the tissues and to produce toxins.(Table 1.3).

Type of virulence factor	Examples	Genes	Associated clinical syndromes
Adhesion to host cells	MSCRAMMs (clumping factors, fibrinogen-binding proteins, collagen and bone-sialoprotein-binding proteins	*clfA, clfB, fnbB, cna, sdr,bbp*	Endocarditis, osteomyelitis, septic arthritis, prosthetic device and catheter infections, nasal colonisation.
Persistence	Biofilm accumulation (polysaccharide intercellular adhesion),intracellular persistence	*ica locus, hemB* mutation	Relapsing infections, cystic fibrosis.
Evading immune defences	Leukocidins (PVL), capsular polysaccharides (5 and 8), protein A, CHIPS, extracellular fibrinogen-binding protein	*lukS-PV, lukF-PV, hgl, cap5 and 8* gene clusters, *spa*	Invasive skin infections, necrotising pneumonia, abscesses, recurrent furunculosis
Tissue invasion and penetration	Proteases, lipases, nucleases, hyaluronate lyase, phospholipase C and metalloproteases (elastase)	*B8, hysA, hla, plc, sepA*	Tissue destruction, metastatic infections
Toxin mediated disease / sepsis	Enterotoxins (A,B,C.D,G,H), toxic shock syndrome toxin-1, exfoliative toxins A and B, α-toxin, peptidoglycan and lipoteichoic acid	*Sea-q, TSST-1 eta, etb*	Food poisoning, toxic shock syndrome, scalded skin syndrome, bullous impetigo and sepsis syndrome
Poorly defined role	Coagulase, ACME, staphyloxanthin	*Arc* cluster, *opp-3* cluster	

Table 1.2 *S. aureus* virulence factors and their role in human infections.
PVL, Panton-Valentine leukocidin; CHIPS, chemotaxis inhibitory protein of staphylococci; ACME, arginine catabolic mobile element; MSCRAMMs, microbial surface components recognizing adhesive matrix molecules. Adapted from Gordon, R. and Lowy, F., 2008.

Staphylococcus aureus has a great repertoire of virulence factors which share two main characteristics: a virulence factor may have several functions in pathogenesis and multiple virulence factors may perform the same function (Gordon, R. and Lowy, F., 2008). Some of the main virulence factors include the formation of an antiphagocytic microcapsule that allows staphylococci to evade the innate immune system and which has also been associated with the ability to cause abscesses (O'Riordan, K. and Lee, J., 2004). The production of protein A (binds to the Fc portion of immunoglobulins, therefore preventing opsonisation) and the ability to adhere to tissues and prosthetic materials helps to evade the immune system and to cause infections at distant and more difficult sites. In addition to the capacity to remain within the "slime" around prosthetic materials provides a reservoir which is inaccessible to both cells of the innate immune system and to antimicrobial treatment. The production of toxins such as toxic shock syndrome toxin-1 (TSST-1) has been associated with increased virulence and pathogenicity, as well as the presence of pathogenicity islands in some of the more virulent strains of *S. aureus.*

1.6.1 Structure of the *Staphylococcus aureus* cell wall

The bacterial cell wall is a key component of the cell; it provides structural rigidity, with-holds the high osmotic pressure of the cytoplasm, gives shape to the cell and is also a physical barrier to the external environment. Bacterial cell walls differ importantly between Gram-positive and Gram-negative bacteria.

Whereas the Gram-positive bacterial cell wall lies at the outermost layer of the cell, in Gram-negative bacteria it is covered under and additional layer: the outer membrane (Washington, W., et al, 2006).

Gram-positive bacteria have cell walls with a thick peptidoglycan (PGN) layer, in which teichoic acids are anchored and lipoteichoic acids are linked to the cytoplasmic membrane (Draing, C., et al, 2008; Zahringer, U., et al, 2008).

1.6.1.1 Peptidoglycan

The component that gives the most rigidity to the cell wall is PGN. It is composed of a backbone of alternating carbohydrate moieties of N-acetylglucosamine and N-acetylmuramic acid in β-1,4 linkage (Beveridge, T., 1981). Short tetrapeptides which are composed of identical short chains of D and L aminoacids are attached to the N-acetylmuramic acid residues through a peptide bond to the lactyl group on C3. Some of these tetra peptides are linked to one another by short peptides forming cross-bridges between adjacent PGN strands. The degree of this cross-linking determines whether the cell wall structure is “tight” (highly cross-linked) or loose. There are variations in the cross-linking of the peptidoglycan among bacteria (Vollmer, W., et al, 2008).

In *S. aureus*, most of the N-acetylmuramic acid residues are cross-linked to adjacent peptidoglycan strands by a cross bridge of five glycine residues providing a rigid cell wall structure. The biosynthesis of the bacterial cell wall is

a continuous process; new peptidoglycan polymers are exported from the cell and linked to pre-existing cell wall polymers at the inner aspect of the cell wall by penicillin binding proteins (PBP) (Paul, T., et al, 1995). These proteins catalyse the elongation of the glycan strands (glycosyltransferase activity) and the interconnection of the glycan strands by peptide cross-linking (transpeptidase activity) (Goffin, C. and Ghuysen, J., 2002; Massova, I. and Mobashery, S., 1998; van H., 2006). The loss of this catalytic activity impairs the ability of the bacterium to control the integrity of the cell wall and the end result is cell lysis. β-lactam antibiotics exert their bactericidal activity by binding to the PBPs and inhibiting polymer renewal at the inner core of the cell wall (Sugai, M., et al, 1997).

Staphylococcal peptidoglycan has been shown to stimulate the production of pro-inflammatory cytokines and chemokines (TNF-α, IL-1β, IL-6 and IL-8) in human monocytes and macrophages (Heumann, D., et al, 1994; Timmerman, C., et al, 1993). The glycan strands are crucial for cytokine production, whereas the stem peptide structure is not (Myhre, A., et al, 2004; Yoshimura, A., et al, 2000).

Studies by Iwaki and colleagues demonstrated that staphylococcal peptidoglycan binds strongly to the soluble recombinant form of the extracellular domain of TLR2 suggesting that the extracellular TLR2 domain directly interacts with peptidoglycan (Iwaki, D., et al, 2002).

1.6.1.2 Teichoic acids:

Teichoic acids are highly charged polymers; they concentrate cations at the cell wall surface. They are involved in activities such as cation balance at the cell surface, cell division and regulation of PGN autolysis. Lipoteichoic acid (LTA) is the main teichoic acid and protrudes from the cytoplasmic membrane through the peptidoglycan layer. It consists of 25 poly (1-3)-glycerol phosphate linked to a diacylglycerolipid anchor (Draing, C., et al, 2008; Zahringer, U., et al, 2008).

S. aureus LTA has been shown to induce the secretion of cytokines and chemokines such as TNF, IL-1β, IL-10, IL-8, IL-12 from monocytes and macrophages (Danforth, J., et al, 1995; Standiford, T., et al, 1994; von AS, et al, 2004; von AS, et al, 2007). Von Aulock and colleagues found that LTA from *S. aureus* was a more potent inducer for the chemokine IL-8 in 160 healthy individuals in comparison to LPS from *Salmonella abortus equi* (von AS, et al, 2006). When comparing the potency of LTA and LPS from Gram-negative bacteria Kusunoki and coworkers noted that very large amounts of LTA were needed to induce inflammatory responses *in vitro*. Concentrations of 1 to 10μg/ml of LTA produced responses equivalent to those produced by LPS concentrations in the ng/ml range (Kusunoki, T., et al, 1995). Nevertheless, comparison of the activity of LPS and LTA demonstrated by Von Aulock, showed that staphylococcal LTA promotes the same strong induction of chemoattractants (IL-8, MIP-1α, MCP-1) as LPS, whereas it was a weaker

inducer of TNF-α, IL-1β and IL-6. They also noted that the cytokine pattern produced by LTA is similar to that induced by the whole bacterium (Von AS, et al, 2004; Von AS, et al, 2007).

1.6.2 Nasal colonisation by S. aureus

Undoubtedly one of the main reasons why *S. aureus* is such a successful organism is its ability to colonise its host. There is evidence that colonisation is the preceding step to infection and patients who are colonised usually develop infection with their same colonising strain (Dall'antonia, M., et al, 2005; Von E., et al, 2001; Watanabe, H., et al, 2000). *S. aureus* colonisation tends to happen predominantly in the anterior nares, although other sites such as the axilla, perineum, throat and gastrointestinal tract are also important reservoirs for the bacterium and should be considered for infection control purposes (Boyce, J., et al, 2005; Gordon, R. and Lowy, F., 2008; Wertheim, H., et al, 2005).

The best studied, has been the role of nasal colonisation. *Staphylococcus aureus* nasal carriage tends to happen in the anterior nares in the vestibulum nasi, which consists of fully keratinised epithelium, apocrine sweat glands, sebaceous glands and hair follicles (Gluck, U. and Gebbers, J., 2000; Wertheim, H., et al, 2005). Several studies have reported the epidemiology of *S. aureus* nasal carriage: 20% of the population are persistent carriers (range 12-30%), 30% are intermittent carriers (range 16-70%) and 50% are non-carriers (range 16-69%). There is

evidence that persistent carriers tend to have a higher bacterial load and are at higher risk of *S. aureus* infection (Wertheim, H., et al, 2005). The increase in the prevalence of MRSA infections and the fact that the bacterium can also be carried asymptomatically in the nose have attracted a great deal of interest due to the implications for clinical practice as well as for the infection control arena (Hidron, A., et al, 2008; Woodford, N. and Livermore, D., 2009).

A recent study by Datta and Huang has shown that persistent carriers who have carried MRSA for more than one year are at high risk for subsequent infection and associated MRSA mortality, particularly within the first three months after colonisation (14%) after which the risk decreases approximately to 4% for the subsequent months (Datta, R. and Huang, S., 2008). In addition the same investigators have previously shown a 33% increase in the risk of developing new MRSA invasive disease within one year, followed by 27% during the second year and 16% thereafter (Huang, S., et al, 2006).

Multiple host factors as well as organism-related factors play a role in colonisation. Host factors include local nasal phenotypic differences: a study by Cole et al demonstrated differences in antimicrobial activity in the nasal secretions of colonised versus non-colonised subjects (Cole, A., et al, 1999). The actual state of competence of the immune system also seems to play a key role in colonisation status, since there is evidence of increasing carriage rates in groups of patients who are considered immunocompromised such as patients with renal impairment

on haemodialysis (Kaplowitz, L., et al, 1988; Kluytmans, J., et al, 1997; Pujol, M., et al, 1996), diabetes mellitus, end-stage liver disease and cancer (Datta, R. and Huang, S., 2008; Kluytmans, J., et al, 1997), as well as infection with HIV (Kluytmans, J., et al, 1997; Weinke, T., et al, 1992). Genetic studies looking for a relationship with the colonisation state have provided conflicting data. Whereas Moore could not find an association between TLR2 gene polymorphisms and susceptibility to severe staphylococcal infections (Moore, C., et al, 2004), the study by Gonzalez-Zorn found that TLR-2 deficient mice developed persistent nasal colonisation (Gonzalez-Zorn, B., et al, 2005). Studies in monozygotic twins have also failed to demonstrate a clear genetic relationship, indicating once more the multifactorial nature of this highly dynamic process (Aly, R., et al, 1974; Hoeksma, A. and Winkler, K., 1963). Other implicated factors are ethnic origin (demonstrating higher carriage rates in white people) (Cole, A., et al, 2001; Williams, R., 1963), age (Armstrong-Esther, C., 1976; Parnaby, R., et al, 1996; Peacock, S., et al, 2003) and previous skin infections with *S. aureus* as well as skin diseases such as eczema and psoriasis (Wertheim, H., et al, 2005; Williams, J., et al, 1999). Host factors are still in need of further research, if we are to improve current clinical practice. This was demonstrated by the study from Wertheim et al in which nasal *S. aureus* carriage and the development of bacteraemia and mortality were followed in non surgical hospitalized patients. They showed that among bacteraemic patients, the non-carriers had higher mortality in comparison to carriers and since most infections in the colonised patients happened with their own colonising strains, therefore, the colonisation status itself seemed to provide

some protective immunity (Wertheim, H., et al, 2004). Humoral immunity also appears to have a protective effect particularly in the case of staphylococcal toxic shock syndrome, which appears to occur only in patients who lack antibodies against the particular toxin during the time of their acute illness (McCormick, J., et al, 2001).

Staphylococcus aureus is very versatile and therefore possesses an arsenal of bacterial factors that support its role as a successful colonising microorganism. Microbial surface components recognising adhesive matrix molecules (MSCRAMMs) are important in colonisation efficacy (Van, B., 2006). Their expression occurs during logarithmic growth, whereas the secretion of proteins happens during the stationary phase. During the infective process, the early expression of MSCRAMMs helps initial colonization into tissues, whereas toxin production allows the spread of the bacterium (Gordon, R. and Lowy, F., 2008). Recent research looking into this aspect of bacterial colonisation has demonstrated the presence of two Staphylococcal proteins implicated in adherence to nasal epithelial cells: clumping factor B (ClfB) and the *S. aureus* surface protein G (SasG). For ClfB in particular, there is evidence of specific binding to human cytokeratin 10 from desquamated nasal epithelial cells (O'Brien, L., et al, 2002). When Wertheim inoculated volunteers with a wild-type *S. aureus* strain and its isogenic strain lacking the ClfB gene, they found that the mutant strain was eliminated faster in comparison to the wild-type strain and persisted up to the end of the follow up in their study. This research highlighted ClfB as a potential

therapeutic target for future decolonisation studies (Wertheim, H., et al, 2008). Roche also showed that SasG promotes bacterial adherence to human desquamated nasal epithelial cells, contributing to establishment of nasal colonisation (Roche, F., et al, 2003).

The increasing rate of MRSA infections prompted the development of new research into nasal colonisation with MRSA. The study by Dall'antonia highlighted the competition for the niche that occurs between MRSA and MSSA strains (Dall'antonia, M., et al, 2005) while Huang and Platt have also observed a 28% rate of relapse within 18 month period after previous infection or colonisation with MRSA (Huang, S. and Platt, R., 2003). The whole dynamics of the colonisation process remains poorly understood, especially due to the lack of study into primary epithelial cells in the subjects who are colonised and their reaction to bacterial ligands.

1.7 Hypothesis:

The respiratory tract is exposed to a multitude of infective, as well as, non infective antigens on a daily basis. Looking more specifically into the infective process, there is evidence that the upper respiratory portion is colonised with bacterial microorganisms such as *S. aureus*, without eliciting an immune response (Watanabe, H., et al, 2000). On the other hand, the presence of the very same pathogen in the lower portion: alveolar compartment is associated with an exuberant pro-inflammatory response which is clinically observed during the development of pneumonia. Furthermore, the presence of *S. aureus* in the nasal compartment is considered a risk factor for developing lower respiratory tract infection with the same bacterial strain. In this manner, the upper respiratory tract acts as a bacterial reservoir (Watanabe, H., et al, 2000).

Although the precise mechanism for this phenomenon has not been researched before, the hypothesis that I want to test is that: ***"there are differences in innate immune regulation, which are responsible of the development of tolerance to S. aureus in the human nasal epithelium, but induce a florid inflammatory response in human alveolar epithelium".***

From this main hypothesis I will attempt to address further specific aspects such as:

- ***"nasal epithelial cells have a lower intrinsic capacity than alveolar epithelial cells to mount an inflammatory response to S. aureus"***
- ***"Tollip is expressed in the human respiratory tract"***
- ***"The nasal epithelium plays an active role in the surveillance of microorganisms"***

1.8 Aims and Objectives of thesis

Arising from the clinical observation described above: that the upper respiratory tract particularly its nasal compartment allows the growth of bacteria, whilst its lower portion reacts with an exuberant inflammatory response to the same microorganisms, the main question is to assess if these differences can be reproduced in an *in vitro* system; using first cell lines representative of both cell types and then to try to translate the findings onto primary cells of the respiratory system in an effort to elucidate the molecular mechanisms underlying these responses.

To assess if tollip is present in the respiratory system, to characterise its role and to try to establish compartmental differences that could explain the induction of tolerance in the upper portion of the respiratory tract

To determine the local epithelial response during nasal carriage of *S. aureus*.

CHAPTER TWO

MATERIALS AND METHODS

2 Materials and Methods

2.1 Cell lines

2.1.1 RPMI 2650 cells

The human nasal cell carcinoma RPMI 2650 was obtained from LGC Promochem (ATCC Number: CCL-30). This cell line was established in 1962 by Moore and Sanberg from a patient with an anaplastic squamous cell carcinoma of the nasal septum (Moore, G., and Sandberg, A., 1964). They reported to have grown it for 35 passages without alteration of the karyotype. The cell line was submitted to the American Type Culture Collection in the 15^{th} passage in 1963 and was grown up to passage 22^{nd}. The karyotype was analyzed and it was noted to be nearly-normal with a stable chromosomal number (Moorhead, P., 1965), characteristic which makes this cell line unique among the human cell lines available at present.

The cells were cultured in Minimum Essential Medium Eagle (MEM) (Sigma Cat No. M2279) with Earle's Balanced Salt Solution adjusted to contain 1.5 g/L sodium bicarbonate supplemented with 10% foetal bovine serum, 1% L-glutamine, 1% non essential amino-acids, 1% sodium pyruvate. Cells were split every three days or when confluence was reached using trypsin/ethylene diamine tetra-acetic acid (EDTA) solution. For experiments, cells were seeded at 2×10^6 in 6-well plates and not used until confluent. Once the monolayer was established, cells were exposed either to a) live *Staphylococcus aureus* (wild-type strain

Newman or isogenic mutant strains deficient for the genes *clumping factor B (clfB)* and *protein A*) or to b) bacterial products derived from *Staphylococcus aureus* such as peptidoglycan (PGN; Fluka – Sigma Aldrich Cat No.77140), lipoteichoic acid (LTA; Sigma Aldrich Cat No L2515), ultrapure *Pseudomonas aeruginosa* lipopolysaccharide (a kind gift from Professor Ian Poxton, University of Edinburgh) and recombinant human tumor necrosis factor Alpha (TNF-α R&D Systems Cat No.210-TA).

2.1.2 T84 cells

The human colon cell carcinoma T84 was obtained from LGC Promochem (ATCC Number: CCL-248). This cell line was derived from a lung metastasis of a colon carcinoma in a patient. The tumour tissue was transplanted in BALB/c nude mice and the cell line was established after 23 passages (Murakami, H. and Masui, H., 1980). Dharmsathaphorn and colleagues noted that the cells form tight junctions and desmosomes and suggested it, as a model for the study of electrolyte transport (Dharmsathaphorn, K., et al, 1984).

The cells were cultured in a 1:1 mixture of Dulbecco's Modified Eagle's Medium (DMEM) / Nutrient Mixture F-12 Ham (Sigma Aldrich Cat No. D6421); supplemented with 5% foetal bovine serum. Cells were cultured and split every four days or when confluent and subcultured using trypsin/EDTA solution and subcultured. For experiments, the cells were seeded at 5×10^5 in a 24-well tissue

culture plate (Costar) and allowed to reach confluence. They were then lysed prior to RNA extraction (see below).

2.1.3 A549 cells

The human type II pneumocyte-like cell line A549, was established in 1972 by Lieber and colleagues from a human alveolar cell carcinoma. They propagated it *in vitro* continuously for more than three years and observed similarities with type II alveolar epithelial cells (Lieber, M., et al, 1976).

The cells were cultured in DMEM (PAA Laboratories Cat No.E15-009) supplemented with 10% foetal calf serum and 1% L-glutamine. A549 cells were split every two to three days and split using trypsin/EDTA as described above. For experiments the cells were seeded at 2 x 10^6 in 6 well plates and once the monolayer was established, they were exposed to live bacteria or bacterial products as described above.

2.2 Isolation of Primary epithelial cells:

2.2.1 Isolation and culture of Primary nasal epithelial cells:

Healthy volunteers and elective patients undergoing pulmonary lobectomy or pneumonectomy in the Cardiothoracic Surgery Department of the Royal Infirmary of Edinburgh Hospital were approached. Those providing informed written consent were enrolled. Ethical approval was granted by the Lothian NHS

Research Ethics Committee (Ref 08/S1102/32). Volunteers or patients were asked to blow their nose before starting the procedure. A nose swab was obtained, using a sterile cotton bud lightly moistened in 0.9% sterile saline (Baxter). Under direct visualisation using a nasal speculum (Instrapac Thudicum Cat No.7861, Medisave, Dorset, UK), the inferior turbinate of the nose was gently brushed using a sterile cytology brush (Surgipath, Cat No. 01970, Peterborough, UK). Both nostrils were brushed. The material obtained was disaggregated and suspended in 2ml of Bronchial Epithelial Growth Medium (BEGM, Lonza, Clonetics) supplemented with 0.1% hydrocortisone, 0.1% recombinant human insulin, 0.1% recombinant human epidermal growth factor, 0.4% bovine pituitary extract, 0.1% retinoic acid, 0.1% gentamicin sulphate and amphotericin B, 0.1% triiodothyronine (T3), 0.1% transferrin and 0.1% epinephrine (Lonza, Clonetics Cat No.CC-3170), and seeded within a single well of a 6-well culture plate previously coated with type I rat-tail collagen (Sigma-Aldrich Cat No.C3867-1VL). After 24 hours, the supernatant and unattached cells were removed and replaced with fresh medium. The removed supernatant was seeded into a second collagen-coated plate to encourage further attachment and growth. Culture media was changed every three days and cells were incubated at 37^{o}C in 5% CO_2 and cultured until 90-100% confluent. Upon reaching confluence, cells were washed with 2ml of 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES)-buffered saline solution (Lonza, Clonetics Cat. No CC-5022), detached using 2 ml of trypsin-EDTA solution (Lonza, clonetics Cat No.CC-5012) and incubated for 10 minutes at 37^{o}C.

Trypsin activity was quenched using 4ml of trypsin neutralising solution (Lonza, Clonetics Cat No. CC-5002). The cell suspension was centrifuged at 279g for 5 minutes at 20^{o}C. The resulting pellet was resuspended in 6 ml of BEGM. Cells were counted and seeded at a density of approximately 6 x 10^5 cells/ml onto an uncoated 6-well plate (costar). All further assays were performed on cells at second passage. If cells were unsuccessful in reaching confluence by the third week, acquired an infection with bacteria or fungi, or if they failed to attach to the collagen within two weeks, they were discarded.

2.2.2 Isolation and culture of primary bronchial epithelial cells:

Cells were obtained from the same group of consented patients as described above, ie undergoing partial lobectomy or pneumonectomy at the Cardiothoracic Surgery Department of the Royal Infirmary of Edinburgh Hospital. After the induction of anaesthesia and endotracheal intubation a broncoscopy brush (Olympus BC-202D Cytology brush Cat No. BC-202D-2010) was inserted blindly through the lumen of the endotracheal tube corresponding to the lung on which no surgery was to be performed. Blind brushings were performed and the material obtained was inoculated immediately in 6ml of BEGM culture medium and transported to the laboratory. Three ml of the cellular suspension were then seeded onto wells coated with type I collagen as described previously in section 2.2.1 and allowed to grow and achieve confluence. All further assays were

performed under identical conditions as for the primary nasal epithelial cells. All the cells were used at passage two.

2.2.3 Isolation and culture of primary type II alveolar epithelial cells

Patients undergoing pulmonary lobectomy or pneumonectomy in the Cardiothoracic Surgery Department under the surgical team led by Mr. William Walker were consented. Ethical approval was given by the Lothian NHS Research Ethics Committee (Ref 08/S1102/32).

Immediately after surgical removal, tissue was transported to the Pathology Department based at the Royal Infirmary of Edinburgh where the duty pathologist removed an appropriately considered portion of macroscopically non diseased lung tissue under strict aseptic technique. The tissue was then immersed in transport medium (0.9% NaCl, 0.5% gentamicin, 1%penicillin/streptomycin and 0.5% amphothericin B). The tissue was allowed to refrigerate overnight and processed the next day. The following day the tissue was injected with 0.9% saline to remove alveolar macrophages, followed by two digestion steps using 0.25% trypsin (Sigma Aldrich Cat No. T8003) in Hanks Balanced Salt Solution (HBSS)(PAA Cat No.H15-009) at 37^{o}C for 15 minutes (10ml per 5 cm^{3} of tissue). Once the digestion was completed, the tissue was cut into small pieces of approx 1-2 mm^{3} in the presence of newborn calf serum (Invitrogen, Cat No, SKU#26010-074), DNAse I (Sigma Aldrich Cat No DN25) was added to the

suspension at 250μg/ml in 7 ml of HBSS and shaken vigorously for 5 minutes before filtering through a large sterile metallic mesh (approx 500μm), followed by a 40μm Falcon cell strainer (BD Biosciences). The filtered suspension was centrifuged at 300g for 10 minutes at 4^{o}C. After centrifugation, the resulting pellet was resuspended in 15ml HBSS / 15ml DCCM-1 (Biological Industries Cat No. 05-010-1) without serum containing 100μg/ml of DNAse I. The suspension was incubated in a T-75 flask (Costar) for two hours at 37^{o}C in a 5% CO_2 atmosphere to allow residual contaminating macrophages to adhere. After two hours, the supernatant containing non-adherent cells was removed and centrifuged at 300g for 10 minutes at 4^{o}C. The pellet was resuspended in complete medium (DCCM-1, 10% Newborn Calf Serum, 1% glutamine, 1% penicillin and streptomycin). This suspension was incubated for two hours at 37^{o}C to allow contaminating fibroblasts to adhere. After two hours incubation the non-adherent cells were removed and centrifuged at 300g for 10 minutes at 4^{o}C. Cells were resuspended in complete medium at 2 x 10^{6} cells/ml and seeded in 6- or 12-well dishes pre-coated with type I bovine collagen Purecol (Nutacon Cat No.5409). The cells were allowed to adhere at 37^{o}C for 24 hours, the medium and any remaining contaminating cells were removed and new fresh medium was added. Cells were re-incubated at 37^{o}C/ 5%CO_2 and after 16 hours the medium was removed and the cells were washed with HBSS. Fresh medium was added and the cells were incubated further to allow establish confluent monolayers with type II pneumocyte morphology. All cells were used at passage 1.

2.3 Bacterial strains

The Gram positive bacterium *Staphylococcus aureus* strain "Newman" was a kind gift from Dr. Ross Fitzgerald from the Centre for Infectious Diseases (CID), University of Edinburgh. This strain was originally isolated from a case of osteomyelitis from a man (Duthie, E. and Lorenz, L., 1952), and has been fully sequenced recently by Baba and colleagues (Baba, T., et al, 2008).

Staphylococcus aureus strain Newman was cultured on tryptic soya broth (TSB), brain heart infusion (BHI) broth and MEM culture media; a growth curve showed that the bacterium grows optimally in TSB and BHI media, but growth on MEM was slightly reduced. Isogenic mutants of this strain were also given by Dr. Ross Fitzgerald, such as a strain lacking clumping factor B (*δClfB*) (Walsh, E., et al, 2008) and a strain deficient in protein A (*δProt A*) (Guss, B., et al, 1985) were used in order to investigate the role of these virulence factors in establishing infection. The MRSA strain 252 was also used, this is a representative strain of the epidemic MRSA-16 clone (EMRSA-16), which has sequenced by Holden and collaborators (Holden, M., et al, 2004). The mentioned strains were cultured in identical conditions to those described for the Newman strain.

2.4 Characterisation of the cell line RPMI 2650

2.4.1 Confocal Microscopy

Confocal microscopy was used to characterise the cell line RPMI 2650. Antibody staining was used to assess the phenotype of the cell line. Antibodies used included those against cytokeratin 7-17 (Zymed Invitrogen Cat No.39-4500), cytokeratin 10 (Zymed Invitrogen Cat No.39-5300), cytokeratin 18 (Zymed Invitrogen Cat No18-0158), and vimentin (Zymed Invitrogen Cat No.18-0052). See Table 2.1.

Antibody	Species	Cat No	Supplier	Concentr µg/ml
Cytokeratin 7-17	Monoclonal mouse anti-human IgG1	39-4500	Invitrogen	20
Cytokeratin 18	Monoclonal mouse anti-human IgG1	18-0158	Invitrogen	20
Cytokeratin 19	Monoclonal mouse anti-human IgG2a	08-0190	Invitrogen	6.5
Cytokeratin 10	Monoclonal mouse anti-human IgG1	39-5300	Invitrogen	20
Vimentin	Monoclonal mouse anti-human IgG1-kappa	18-0052	Invitrogen	1
Tollip	Polyclonal rabbit anti-human IgG	Ab37155	Abcam	1
Isotype control mouse	Mouse IgG, IgM	SKU# 08-6599	Invitrogen	0.5
Isotype control rabbit	Rabbit IgG	SKU# 08-6199	Invitrogen	0.5
TLR-1 (CD281)	Monoclonal mouse anti-human IgG1	SM1828P	Acris Antibodies	20
TLR-2	Monoclonal mouse anti-human IgG2a	MHTLR 200	Invitrogen	20
TLR-4	Rabbit polyclonal anti-human IgG	36-3700	Invitrogen	10
TLR-6	Monoclonal mouse anti-human IgG1-kappa	39-0800	Invitrogen	20
TLR-9	Monoclonal mouse anti-human IgG1	OP185	Calbiochem	20

Alexa Fluor 488	F(ab')2 fragment of goat anti-mouse IgG	SKU#A-11017	Invitrogen	0.005
Alexa Fluor 488	Goat anti-rabbit IgG	SKU#A-11008	Invitrogen	0.005
Alexa Fluor 488	Goat anti-mouse IgM (μ chain)	SKU#A-21042	Invitrogen	0.005

Table 2.1 List of antibodies used for immunocytochemistry and confocal microscopy

Cells were seeded at 5 x 10^5 in glass coverslips in individual wells of a 6-well plate and allowed to adhere for 15 minutes prior to adding 2ml of culture medium and incubated overnight. The following day, 1 ml of medium was removed, 1 ml of ice cold methanol was added, and the plates were incubated on ice for 10 minutes. The coverslips were washed three times with phosphate buffered saline (PBS) and blocked with 2ml of 2% goat serum for 30 minutes. The coverslips were dried and placed in an incubation chamber and incubated with the corresponding antibody at appropriate concentration overnight, taking care to avoid evaporation of the antibody solution. The next day, the coverslips were washed three times with PBS, and the secondary antibody (see Table 2.1) was added at a 1:200 dilution, and incubated at room temperature for 1 hour. The coverslips were washed again three times with PBS and dried and mounted using 50μl of Vectashield with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories Cat No. H-1500). The coverslips were visualized the following day using the Leica TCS SP5 confocal microscope (Leica Microsystems CMS

GmbH) based at the Confocal and Advanced Light Microscopy (CALM) facility in the Queen's Medical Research Institute (QMRI).

2.5 Characterisation of primary epithelial cells:

2.5.1 Primary nasal cells:

Primary nasal epithelial cells were characterised by immunocytochemistry. Once cells reached confluence they were detached using 2ml of trypsin/EDTA solution applied at 37^{o}C for 10 minutes followed by centrifugation. Cells were resuspended then, seeded onto sterile glass coverslips and processed as described above in section 2.4.1. Cells were tested for cytokeratin 7-17, 18 and 19, as well as vimentin, using the same antibodies described in section 2.4.1 and table 2.1.

2.5.2 Primary type II alveolar epithelial cells:

Type II alveolar epithelial cell phenotype was confirmed by positive staining for alkaline phosphatase (Dobbs, L., 1990) and reverse transcriptase – polymerase chain reaction (RT-PCR) for Surfactant Protein-C (SP-C) and aquaporin 3 (AQP3) (Fuchs, S., et al, 2003; Armstrong, L., et al, 2004).

2.5.2.1 Alkaline Phosphatase Stain

Primary type II alveolar epithelial cells were phenotyped by staining for alkaline phosphatase (ALP). Ten mg of naphthol-AS-bisphosphate (Sigma Aldrich Cat No. N2125) was dissolved in 40µl of dimethyl sulphoxide (DMSO) and added to 10ml of alkaline phosphatase buffer (0.625M magnesium chloride and 0.125M

2-amino-2-methyl-1- propanol; pH: 8.9). Then 10mg of fast red violet LB salt (Sigma Aldrich Cat No.F3381) was added and the solution was mixed for 5 minutes at room temperature. This was then filtered through a 0.22μm filter and used immediately. 1ml was added to the monolayers and the cells were incubated for 20 minutes at 37^{o}C. Photographs were taken using a light microscope.

2.5.2.2 Reverse transcriptase-Polymerase Chain Reaction for Surfactant Protein-C (SP-C) and Aquaporin 3 (AQP3)

Total RNA was extracted as it will be described in detail in section 2.8 and 1μg was reverse transcribed. Primers used were described from the paper from Armstrong et al, 2004 for *SP-C:* forward primer: AGCAAAGAGGTCCTGATGGA; reverse primer CTAGTGAGAGCCTCAAGACT, for *AQP-3:* forward primer: CCTTTGGCTTTGCTGTCACTC and reverse primer: ACGGGGTTGTTGTAAGGGTCA.

PCR was performed under the following conditions: DNA was denatured at 94^{o}C for 3 minutes, followed by 30 cycles of 94^{o}C for 20 seconds, annealing at 54^{o}C for 45 seconds and extension for 72^{o}C for 45 seconds. Final extension was performed at 72^{o}C for 5 minutes. Samples were then run on a 2% agarose gel and then visualised using the Molecular Imager Versadoc MP4000 System PC (Bio-Rad

Laboratories, Inc). Negative controls for the PCR reaction included a non reverse transcriptase control as well as RNA extracted from the cell line A549 (Witherden, I., et al, 2004; Armstrong, L., et al, 2004). *GAPDH* was the house keeping gene used for this, primers used were the same as the ones that will be described in section 2.8.

2.6 Constitutive cytokine production in cultured supernatant by cell lines

The cell lines RPMI 2650 and A549 were seeded at 5 x 10^5 cells/ml on a 24-well plate (Costar) once confluent, supernatants were removed at 0, 2, 4, 6 and 24 hours. Supernatants were stored at -80^{o}C prior to enzyme-linked immunosorbent assays (ELISAs). Cytokines measured included transforming growth factor-β2 (TGF-β2) (R&D Systems Cat No.DY302), interleukin-8 (R&D Systems Cat No. DY208), interleukin-6 (R&D Systems Cat No DY206) and interleukin-10 (R&D Systems Cat No.DY217B), following the manufacturers instructions.

Total protein concentration was measured in cell lysates using the BCA TM® protein assay kit (Thermo scientific USA, Cat No 23227), following the manufacturer instructions. This was done in order to permit normalisation of the ELISA assay to the total protein content for each individual sample.

2.7 Bacterial co-cultures:

The cell line RPMI 2650 was seeded at a density of 1 x10^6 cells per well in a 24-well plate. The same day 5ml of MEM were inoculated with one colony of *Staphylococcus aureus* strain Newman and incubated overnight at 37^{o}C, rotating at

250 revolutions per minute (rpm). The following day a fresh 1:200 dilution of the overnight bacterial culture was inoculated in 5ml of MEM and allowed to grow for a further 3 hours in order to ensure that the bacteria used were in the logarithmic phase of growth. After 3 hours in culture, the bacterial suspension was washed 3 times with MEM and resuspended in MEM to achieve an O.D. approximately of 0.1. Appropriate volumes were added to the cells according to the desired multiplicity of infection (MOI) dose and bacterial suspensions were also plated on to tryptic soy agar (TSA) to perform viable counts the following day for further assessment of the bacterial dose used.

The cells were washed twice with MEM and infected with two different doses of bacteria: 1 x 10^5 cfu/ml and 1.6 x 10^5 cfu/ml. After infection, the cells were incubated for a further 4 or 24 hours at 37^{o}C/5% CO_2 and samples were processed for cell lysis and RNA extraction (as will be described in section 2.9). Experiments were performed using the cell line A549 under identical conditions as described above.

2.8 Development of an in-house tollip ELISA

Recombinant human tollip protein was purchased from Abnova Cat No. H00054472-P01 Taiwan: Full length protein (275 a.a.) with a GST tag in a buffer with 50mM Tris-HCl, 10mM reduced glutathione, pH8.0. The recombinant protein was produced by a wheat germ cell-free protein synthesis system. The purity of the received protein was 90%. Ten microlitres of a stock solution of

tollip (initial concentration 100μg/ml) were diluted in 990μl PBS and serial dilutions performed to give an eight point standard curve (1000ng/ml, 500ng/ml, 250ng/ml, 125ng/ml, 62.5ng/ml, 31.25ng/ml, 15.6ng/ml and 0ng/ml). Four different anti-tollip antibodies were tested in order to develop the assay: antibody 1: mouse, anti-human tollip monoclonal antibody (MO1), unconjugated, clone 1A5-2A3 (Abnova corporation, Cat No.H00054472-MO1), antibody 2: rabbit anti-human polyclonal antibody to tollip (Abcam, Cat No.ab56202), antibody 3: mouse anti-human tollip monoclonal (Kimmy-2) (Abcam, Cat No. ab16089) and finally antibody 4: rabbit anti-human polyclonal to tollip (Abcam, Cat No. ab37155). Best results were achieved using antibody four.

A 96- well plate (Nunc) was coated with 100μl of either standard (human recombinant tollip as described above), or cell lysate and incubated overnight at room temperature. The following day the plate was washed with wash buffer (0.05% Tween 20 in PBS) three times and blocked with 1% bovine serum albumin (BSA) (R&D Systems, Cat No. DY995) for one hour at room temperature. The plate was washed again three times with wash buffer and rabbit polyclonal primary antibody to tollip (Abcam, Cat No. ab37155-100) was diluted in PBS and added at a concentration of 10μg/ml to incubate for 2 hours at room temperature.

The plate was then washed three times with wash buffer and goat anti-rabbit IgG complexed to horseradish peroxidase HRP (Zymax Cat No.62-6120) was added at 1:2000 dilution in PBS with 1% BSA and incubated for 30 minutes at room

temperature. The plate was washed three times and 100µl of Sureblue tetramethylbenzidine (TMB) Microwell Peroxidase Substrate (KPL, Cat No. 520001) reagent were added and incubated for 20 minutes, followed by 100µl of the stop solution 2N H_2SO_4. The plate was then read using the microplate data collection and analysis software, Biotek (Biotek instruments incorporated, Vermont, USA) optical densities were determined at 450nm with a reference filter of 540nm. Results were normalised to total protein content in the samples as previously mentioned in section 2.6.

2.9 Rt-PCR for tollip in cell lines

Total RNA was extracted from T84, RPMI 2650 and A549 cell lines using the Trizol extraction method (Chomczynski, P., and Sacchi, N., 1987). After each experiment, supernatant media was removed and monolayers were washed with PBS, 1 ml of Trizol (Invitrogen Cat No.15596-026) was added and cell lysates were stored at -80^{o}C until assayed. After defrosting, 200µl of chloroform was added and incubated on ice for 5 minutes, followed by centrifugation at 12500g for 15 min at 4^{o}C. After centrifugation 600µl of the clear top aqueous phase were aspirated gently and isopropanol was added. The tube was mixed and placed at -80^{o}C for 1 hour. Tubes were thawed on ice for 10 minutes and centrifuged at 12500g for 20 minutes at 4^{o}C. The supernatant was discarded and 300µl of ice cold 70% ethanol was added and vortexed for 5-10 seconds, followed by centrifugation at 12500g for 10 minutes at 4^{o}C. The supernatant was removed taking care not to disrupt the pellet which was then allowed to dry for 15 minutes

at room temperature. Forty-three μl of RNAse-free water was added to the pellet and the mixture vortexed gently for 20 seconds at room temperature. Samples were then assayed for reverse transcription of RNA using the Improm-II^{IM} System (Improm-II^{TM} Reverse Transcriptase Cat No. A3802, Promega, Madison, USA).

The reverse transcriptase polymerase chain reaction (RT-PCR) was performed using the following primers for *tollip*: forward primer: OM1:TACAGGCAAAGTTGGCCAA (which binds a region of the *tollip* gene between the second and third exons), reverse primer: OM2:AAGCAGCGCGTAGGACAT, (which binds to a region between the fourth and fifth exons). The expected product size was 347 base pairs (bp). Primers were designed with the help of Dr. Thomas Wilkinson using the software Primer3® (Whitehead Institute for Biomedical Research, Steve Rozen and Helen Skaletsky, USA).

Complementary DNA (cDNA) was denatured at 94^{o}C for 3 minutes, annealed at 58^{o}C for 1 minute and extended at 72^{o}C for 1 minute for 35 cycles, and then allowed a final extension step at 72^{o}C for 5 minutes. Samples were run using the PTC-100 Programmable Thermal Controller (MJ Research, Inc, Watertown, MA, USA). After PCR was completed, PCR products were then run on a 2% agarose gel and visualised using ethidium bromide staining on the Molecular Imager Versadoc MP4000 System PC (Bio-Rad Laboratories, Inc).

For the *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* housekeeping gene. Primers were designed using the Primer3 software as above. Forward primer: 5'-CGAGATCCCTCCAAAATCAA-3' and reverse primer: 5'-TGCTGTAGCCAAATTCGTTG-3'. Predicted product size was 727 base pairs. The PCR was performed in the same conditions as per *tollip*, but run for 20 cycles only.

A second PCR reaction was performed using primers published in the literature by Otte, J., et al, 2004. The expected product size was 74 base pairs. The PCR conditions used were the same as above.

2.10 Real-time PCR for Tollip

Total RNA was extracted from primary nasal, bronchial and alveolar type II cells using the total RNA Isolation kit NucleoSpin RNA II (Macherey-Nagel Cat No.740955.50). A total of 1μg of RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Cat No.4368814) following the manufacturer's instructions. Real-time PCR primers and probe for *tollip* were designed with the assistance of Dr. Thomas Wilkinson using the software Primer3® as mentioned previously in section 2.9. Forward primer: 5'-TGGAATAAGGTCATCCACTG-3', reverse primer: 5'-AGAAGGCTCTCTCATCGAA-3', Probe: 5'-CCCAGGCGTGGACTCTTTCTA-

3’. Dissociation curves were used to assess the quality of the primers and to rule out the possibility of having primer dimmers (Vandesompele, J., et al, 2002).

Primers and probe were also designed for the housekeeping gene *GAPDH* using the same software. Forward primer: 5’-GAAGGTGAAGGTCGGAGTC-3’, reverse primer: 5’-GAAGATGGTGATGGGATTTC-3’ and probe: 5’-CAAGCTTCCCGTTCTCAGCC-3’. Primers were tested and dissociation curves were performed to assess specificity and to rule out the presence of primer dimmers as described above. A second house keeping gene for PCR was chosen in order to perform normalisation of the gene of interest results: *18S,* using a TaqMan® endogenous control (Eukaryotic 18S rRNA endogenous control Cat No. 4352930E, Applied biosystems)

Diluted cDNA (1:100) was used as a template for the PCR reaction and samples were loaded onto the Applied Biosystems 7500 Fast Real-Time PCR System. The specificity of the reactions was controlled by using “no-template” and “no-reverse transcription controls”. Results were normalised to the human *18S* gene using the standard curve method (Applied biosystems, 2008).

2.11 Comparison of the Cytokine profile between the cell-line RPMI 2650 and A549

RPMI 2650 and A549 cells were seeded in 6 well plates at 2 x 10^6 cells/ml and allowed to reach confluence. After the monolayers were established, cells were washed with medium (MEM and DMEM, respectively), 3 times and stimulated with 100ng/ml *Pseudomonas aeruginosa* ultrapure LPS (kind gift from Professor Ian Poxton from the Centre for Infectious Diseases), 10μg/ml of *S. aureus* PGN (Fluka, Sigma-Aldrich), 10μg/ml of *S. aureus* LTA or 10ng/ml of human recombinant tumor necrosis factor-alpha (Hr-TNF-α). Cells were incubated for 24 hours and supernatants were removed and stored at -80^{o}C until assayed. Enzyme-Linked Immunosorbent Assay (ELISA) was used to measure cytokines in cell supernatants. Levels of IL-8, IL-6,IL-10 were determined using commercially available ELISA kits (R&D Systems), SLPI levels were determined using the SLPI quantikine ELISA kit (R&D Systems Cat No. DP100). Tollip levels were determined in cell lysates using the in-house ELISA developed protocol as described in section 2.8 above.

2.12 Bacterial identification

2.12.1 Identification of nasal carriage of Staphylococcus aureus

Healthy volunteers and patients (as described in section 2.2.1 above) had a nasal swab performed prior to the isolation of primary nasal epithelial cells. A sterile cotton wool swab was moistened in sterile 0.9% saline and then the anterior

portion of the right nostril was swabbed, followed by inoculation on two separate horse blood agar plates (Oxoid). Plates were incubated at 37^{o}C, on 5% CO_2 atmosphere for 18 to 24 hours. Multiple colonies were separated further into blood agar and chocolate agar plates (Oxoid) and re-incubated. Once plates appeared to have a single predominant type of colony, bacterial identification was performed primarily to investigate the growth of the bacterium *S. aureus*.

All Gram-positive colonies were tested for catalase production, Staphytect test and coagulase production (please see below).

2.12.1.1 Catalase Test:

In order to exclude growth of streptococci (catalase negative), a full colony of the bacterium being grown onto chocolate agar was tested was transferred onto a glass slide and one drop of 3% hydrogen peroxide (Sigma Aldrich Cat No.H-1009) was added. The slide was then observed for "bubble formation": the rapid and sustained appearance of bubbles /effervescence was interpreted as a positive test. The appearance of small bubbles after 20 to 30 seconds was not considered a positive test. *Staphylococcus aureus* strain Newman was used as a positive control and a *Streptococcus* strain was used as a negative control was every time that the test was performed.

2.12.1.2 Latex agglutination test

This diagnostic test for the differentiation of *Staphylococcus aureus* utilises the detection of clumping factor, protein A and some polysaccharides found in

methicillin resistant *Staphylococcus aureus* (MRSA) from those staphylococci that do not possess these properties. It uses latex beads coated with plasma. Fibrinogen bound to the latex detects clumping factor. In addition, immunoglobulin molecules also present in the beads detect protein A (Staphytect plus, Oxoid, Cat No. DR0850 and M43 Microgen^{TM} STAPH, Microgen bioproducts, Surrey, UK). Briefly, a drop of the test reagent and the control reagent were added onto the reaction card, followed by the inoculation of a loopful of a colony to be tested, the suspension was mixed with a sterile loop and then mixed by rocking the test card by hand for 60 seconds observing the development (or not) of agglutination in the sample. Each single reaction was also tested with a known strain of *S. aureus* strain Newman as a positive control.

2.12.1.3 Tube coagulase test

Free coagulase is a thrombin-like substance present in culture filtrates. Once a suspension of coagulase-producing organisms is prepared in plasma in a test tube, a visible clot forms as a result of the coagulase reacting with a serum substance (coagulase reacting factor) to produce the fibrin clot. Eighteen to twenty four hour cultures were tested by adding a loopful of the test strain to a tube containing 500µl of rabbit plasma with EDTA (Animal Biologicals Cat No. IRB-N). The tube was then incubated at 37^{o}C for 4 hours at which time clot formation was observed by gently tilting the tube. If no clot was observed at that time point, the tube was re-incubated at room temperature for a further 18 hours and read again. The test was considered to be positive if a visible clot was observed either at 4 or 24 hours.

A positive control (*S. aureus* strain Newman) was used and a negative control (coagulase negative Staphylococci) were tested each time.

2.13 Toll-Like Receptors in Primary nasal epithelial cells

The expression of TLRs in primary nasal epithelial cells was assessed by immunocytochemistry and confocal microscopy. Primary nasal epithelial cells were seeded into sterile coverslips and processed as described above in section 2.5.1. Antibodies were used against TLR-1, 2, 4, 6 and 9. Primary human monocytes were used as positive controls. They were obtained from consented healthy donors after a separation protocol; they were a kind gift from Dr. HiSin-Ni Li from the Davidson's group, Lung Inflammation group, Centre for Inflammation Research, (CIR), University of Edinburgh. For antibody concentrations and further details please refer to the table 2.1.

2.14 Flow Cytometry of Nasal Brushings

This work is the result of collaboration with Dr. Paul Fitch from the Schwarze/Immunity and chronic inflammation group based at the QMRI, University of Edinburgh.

Healthy volunteers were consented for performing nasal brushings analysed by flow cytometry. Volunteers were asked to clean their nose and a sterile wool swab lightly moisturised in 0.9% saline was used to perform a nasal swab. The swab

was inoculated on a horse blood agar plate as described above in section 2.2.1. Under direct vision of the inferior nasal turbinate a sterile cervical brush was used to obtain nasal cells. The material obtained was resuspended in PBS and centrifuged at 275g for 5 minutes at 4^{o}C. It was then resuspended in ice cold FACS wash fluid (CellWASH, Cat No. 349524, Becton Dickinson biosciences) containing 1% mouse serum, using a syringe and a 19g or 20g needle, the sample was mixed gently until it was deemed to be a homogeneous cellular suspension. The suspension was centrifuged at 275g for 5 minutes at 4^{o}C and the supernatant was discarded, followed by vortexing of the pellet. The cells were resuspended in a final volume of 200µl with FACS wash fluid containing 1% mouse/rat serum and a cell count was performed. A 50µl of cell suspension was added to a black bottom low adherence 96-well plate (Nunc® FluoroNunc™ Cat No. P8741, Sigma), kept on ice and 1:10 dilution of the relevant antibody was added and mixed well. The plate was incubated for 30 minutes on a gently rocking platform at 4^{o}C. After which the plate was centrifuged at 300g for 5 minutes and the supernatant was removed. Two hundred µl of FACS-lysing solution (Becton Dickinson, Cat No349202) was added to the samples and mixed gently. The plate was incubated in the dark for a further 8 minutes at 4^{o}C. After incubation, it was centrifuged at 300g for 5 minutes at 4^{o}C and washed three times with FACS wash solution. Cells were resuspended in 200µl FACS wash, protected from light and stored at 4^{o}C until run in the BD FACScalibur flow cytometer (Becton Dickinson and company©) held at the cytometry facility based at the CIR in the Queen's Medical Research Institute, University of Edinburgh. Once ready for assay,

samples were transferred onto FACS tubes (BD biosciences) containing 100µl PBS. The forward scatter (relative size) verses side scatter (relative granularity) gates were defined using a heat and UV killed Propidium Iodine stained cell control to exclude dead cells.

The antibody panel included antibodies raised against CD16 (FITC labelled-BD Pharmingen, Cat No. 555406), epithelial cell adhesion molecule (EPCAM) (PE labelled-BD Pharmingen Cat No.347198), CD14 (PERCP labelled-BD Pharmingen Cat No. 345786) and CD3 (APC labelled-BD Pharmingen Cat No. 345767).

2.15 Cytokine response by primary nasal epithelial cells

Primary nasal epithelial cells from healthy volunteers and patients were cultured to confluence as described above in section 2.2.1. The cells were used at passage two, once confluent. Monolayers were washed 3 times with fresh BEGM and non stimulated or stimulated with 100ng/ml of *Pseudomonas aeruginosa* ultrapure LPS (kind gift from Professor Ian Poxton from the Centre for Infectious Diseases), 10µg/ml of *S. aureus* PGN (Fluka, Sigma-Aldrich), 10µg/ml of *S. aureus* LTA, 1µM of the oligonucleotide CpG-C DNA prototype ODN 2395 (Hycult biotechnology b.v., Cat No. HC4041) and 10ng/ml of Hr-TNF-α. Cells were incubated for 24 hours at 37oC/5% CO2. Supernatants were removed and stored at -80^{o}C until assayed using the BD^{TM} Cytometric Bead Array (CBA) Human Inflammatory Cytokine kit (BD Biosciences Cat No.551811), which measures IL-

8, IL-6, IL-1β, IL-10, TNF and IL-12p70 using the BD FACSarray at the CIR Flow Cytometry Facility in the QMRI. The results were analysed using GraphPad Prism (Version 5 GraphPad Software, Inc. La Jolla, CA). For *S. aureus* colonization experiments the cytokine concentration was related to the particular colonisation status of each individual subject.

2.16 Cytokine response by primary bronchial epithelial cells

Primary bronchial epithelial cells from patients undergoing pulmonary lobectomy or pneumonectomy were grown to confluence as described above in section 2.2.2 and supernatants were assayed using identical conditions as for the primary nasal epithelial cells.

2.17 Cytokine response by primary type II alveolar epithelial cells

Type II alveolar epithelial cells were harvested as described above in section 2.2.3 and plated onto plates pre-coated with bovine type I collagen. Cells were allowed to adhere to the collagen and then washed with HBSS. Once they achieved confluence (approximately after 5 days), they were washed 3 times with DCCM-1 medium supplemented with L-glutamine, 10% foetal calf serum, 1% penicillin/streptomycin and then stimulated with 100ng/ml *Pseudomonas aeruginosa* ultrapure LPS, 10μg/ml of *Staphylococcus aureus* PGN, 10μg/ml of *Staphylococcus aureus* LTA, 1μM of the oligonucleotide CpG-C DNA prototype

ODN 2395 and 10ng/ml of Hr-TNF-α. Cells were incubated for 24 hours at 37^{o}C and supernatants were removed and stored at -80^{o}C until assayed using the BD^{TM} Cytometric Bead Array (CBA) Human Inflammatory Cytokine kit (BD Biosciences Cat No.551811), which includes IL-8, IL-6, IL-1β, IL-10, TNF and IL-12p70 using the BD FACSarray at the Flow Cytometry Facility in the QMRI. Statistical analysis was performed using the GraphPad Prism software (Version 5 GraphPad Software, Inc. La Jolla, CA).

2.18 TLR-9 stimulation experiments in primary nasal epithelial cells and primary type II alveolar epithelial cells

In order to try to elucidate the role of TLR9 in the innate immune response to bacterial DNA, primary nasal epithelial cells were seeded in a 12-well plate (Costar) until confluence. Once confluent, cells were washed three times with fresh BEGM and then exposed to 1μM of the oligonucleotide CpG-C DNA prototype ODN 2395 (Hycult biotechnology b.v., Cat No. HC4041), 10μM of the inhibitory oligonucleotide ODN TTAGGG (Invivogen Cat No.tlrl-hinhodn) or left unstimulated for 24 hours at 37^{o}C/5% CO_2. Cells were challenged further for 24 hours by the addition of 100ng/ml *P. aeruginosa* LPS, 10μg of *S. aureus* LTA, 1μM of CpG-C DNA or 10μM of ODN TTAGGG. After a total incubation of 48 hours at 37^{o}C supernatants were removed and stored at -80^{o}C for cytokine assays and cells were harvested and lysed for protein analysis.

Type II alveolar epithelial cells were seeded onto wells pre-coated with bovine type I collagen in a 12-well plate and cultured until confluence around day 5 and exposed to bacterial DNA and bacterial products under identical conditions to those described above.

CHAPTER THREE

RESULTS

Results

3.1. Comparison of differences between cells from the respiratory tract

3.1.1. Introduction

The vocal cords represent a landmark demarcating the upper and lower respiratory tract. The lower respiratory tract can be conveniently divided again into the conducting airways (as far as the terminal bronchioles) and gas-exchanging regions of the lung (from the respiratory bronchioles to the alveoli). The use of epithelial cell lines has been widespread in the research into the respiratory tract, particularly since epithelial cell lines are easily accessible, easy to store and have so far provided valuable information regarding the cellular behaviour of nasal as well as lung epithelium. On the other hand, cell lines may not be representative of primary cells from the corresponding region. For example the nasal epithelial cell line RPMI 2650 is derived from a human nasal cell carcinoma (Moorhead, P., 1965) and the type II alveolar epithelial cell line A549 is derived from a lung adenocarcinoma (Lieber, M., et al, 1976) Cancerous cells grow at a very high speed, lack the contact inhibition that normal cells possess and are metabolically more active (Hanahan, D., and Weinbergh, R., 2000, Pan, C., et al, 2009). They are also good at disguising themselves from the immune response in order to be able to proliferate. Wistuba and coworkers have compared 12 types of human non-small cell lung cancer (NSCLC) cell lines with

their corresponding original tumours and found that NSCLC cell lines retain the properties of their parental tumours for long periods of time (Wistuba, I., et al, 1999). In contrast, primary cells are difficult to obtain, very fragile during culture and not easily stored, and have a limited lifespan. Nevertheless these cells are the preferred material in order to establish normal physiological epithelial cell behaviour.

The hypothesis that I would like to test are:

1. ***"nasal epithelial cells have a lower intrinsic capacity than alveolar epithelial cells to mount an inflammatory response to S. aureus"***
2. ***"nasal and alveolar epithelial cell lines mimic poorly primary nasal and alveolar epithelial cells"***

In view of these considerations, my aim was to characterize the phenotype and cytokine response of cell lines considered representative of the upper and lower respiratory tract, and to compare them to primary human epithelial cells of the respiratory tract. For this I used the cell line RPMI 2650 and compared it against primary human nasal epithelial cells. In addition I studied the cell line A549 and compared it against primary human type II alveolar epithelial cells.

3.2. Characterisation of the upper respiratory tract

3.2.1. The cell line RPMI 2650

The human nasal epithelial cell line RPMI 2650 has been described in the past to closely resemble the normal nasal epithelium, especially regarding karyotype

(Moorhead, P., 1965) and cytokeratin polypeptide pattern. It typically grows in clusters both horizontally as well as vertically and does not form neat monolayers *in vitro* (Figure 3.1) (Salib, R., et al, 2005; De Fraissinette, A., et al, 1995; Kasper, M. and Stosiek, P., 1990). On light microscopy the cells were observed to be small in size, with a granular appearance. Rapid growth with a high mitotic rate was also observed, in accordance with the findings of Bai, S., et al, 2008. Although it has been reported by Moore and Sandberg (1963) and Moorhead (1965) that this cell line exhibits the presence of mucoid material at the cell surface I did not observe any evidence of mucus production.

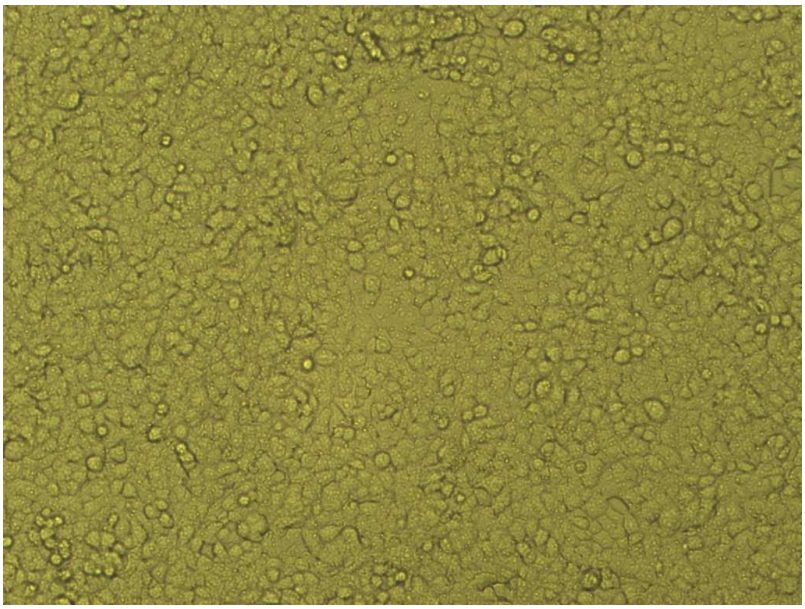
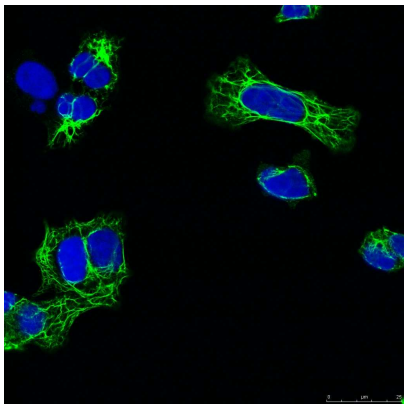


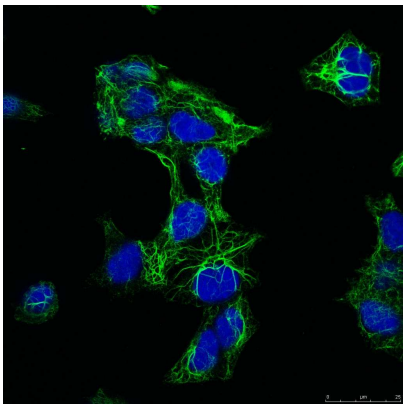
Figure 3.1. Morphology of the human nasal cell line RPMI 2650.
Cells were cultured in MEM supplemented with L-glutamine, non-essential aminoacids, sodium pyruvate and 10% foetal bovine serum in 24 well plates. Cells were plated at $5x10^5$ cells/ml, and photomicrographs were taken 24 hours after initial inoculation of the plate. 650x magnification.

3.2.1.1. Cytokeratin pattern

After establishing the basic culture conditions for the cell line, I proceeded to characterise the cells according to their cytokeratin expression pattern. This was done using immunocytochemistry techniques and confocal microscopy. RPMI 2650 cells displayed low expression of cytokeratin 10 and high levels of cytokeratin 7-17, cytokeratin 18 and vimentin. These findings are in keeping with previous observations (De Fraissinette, A., et al, 1995; Moll, R., et al, 1983). (Figure 3.2)

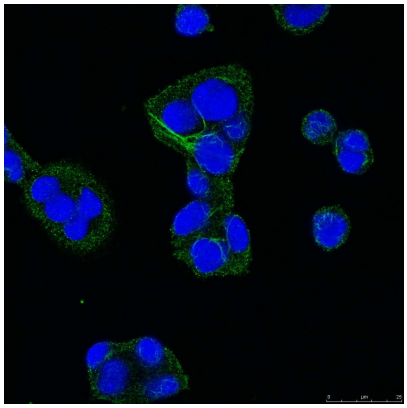
a) CK 7-17

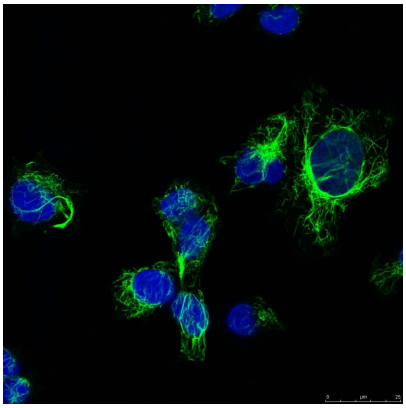




b) CK18

c) CK10





d) Vimentin

e) Isotype control

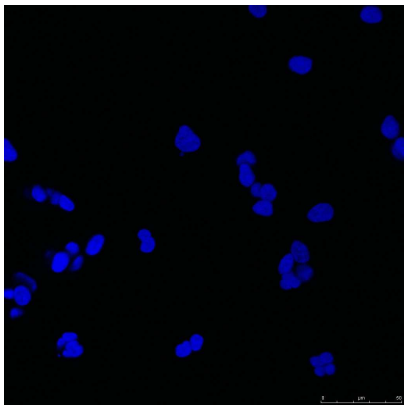


Figure 3.2. Cytokeratin polypeptide pattern of the RPMI 2650 cell line.
RPMI 2650 cells were plated at 2.5 x10^5/ml in coverslips, fixed with methanol, blocked with 2% goat serum and subsequently incubated with murine monoclonal antibodies against a) Cytokeratin 7-17, b) Cytokeratin 18, c) Cytokeratin 10, d) Vimentin e) Isotype control. Nuclei were stained with DAPI (blue). Secondary antibody was anti-mouse IgG Alexa 488(green). Images were analysed using confocal microscopy. Scale bar equals 25µm in detailed images, 50µm for the isotype control. The typical morphology of cytokeratin filaments is observed.

3.2.1.2. Constitutive cytokine production

After the cytokeratin expression confirmed the epithelial phenotype of the cell line, I proceeded to perform functional studies on its constitutive behaviour. The RPMI 2650 cell line has been reported to produce the cytokine TGF-β2 (Salib, R., et al, 2005, Carey, B., et al, 1993). The secretion of TGF-β2 into cell-free supernatants appears to be an active process, levels of this cytokine at 4 hours were 193.6 pg/mg of lysate protein (±73.21) and rose significantly to 516.1 pg/mg of protein (±359.6) at 24 hours ($p<0.05$) (Figure 3.3).

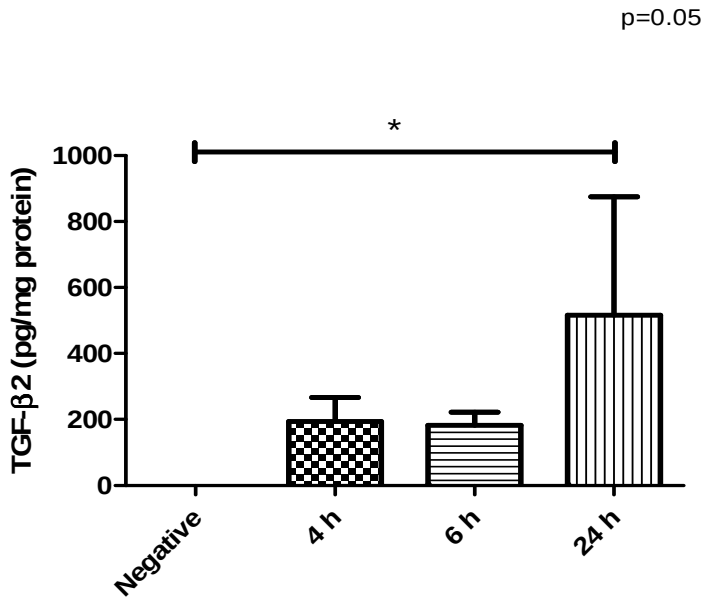


Figure 3.3. TGF-β2 production by the human nasal cell line RPMI 2650 (n=3).
The cells were plated at 5 x10^5 cells/ml in a 24 well plate. Once they achieved 90% confluency, supernatants were removed at different time points and TGF-β2 levels were measured by ELISA and normalised to total protein content in cell lysates. Negative control: cell-free medium
Data are presented as mean (columns) ±SD (error bars). Data analyzed using ANOVA, post test: Dunn's test.
Statistical significance (*) determined when $p<0.05$.

Constitutive levels of the pro-inflammatory cytokines IL-8, IL-6, TNF-α were non detectable in RPMI 2650 cells by ELISA.

Since the characterisation of the cell line RPMI 2650 was in agreement with the findings in the current literature, I proceeded to try to establish an infection model to provide important information regarding the interaction between Staphylococci and nasal epithelium that happens early during the colonisation process.

3.2.1.3. Bacterial and eukaryotic co-culture experiments

Bacterial and eukaryotic co-culture experiments provide useful and valuable information on the real dynamics of eukaryotic-prokaryotic interactions. Since one of the main objectives of this work was to try to investigate how *S. aureus* interacts with the respiratory epithelium and to try to dissect fundamental differences between the responses, eukaryotic and prokaryotic cultures were performed using the nasal cell line RPMI 2650 and the bacterium *Staphylococcus aureus* strain Newman.

S. aureus was grown in TSB and MEM culture media. A growth curve showed optimal growth in TSB. Growth on MEM, despite being slightly lower, was almost comparable to the other media used. (Figure 3.4)

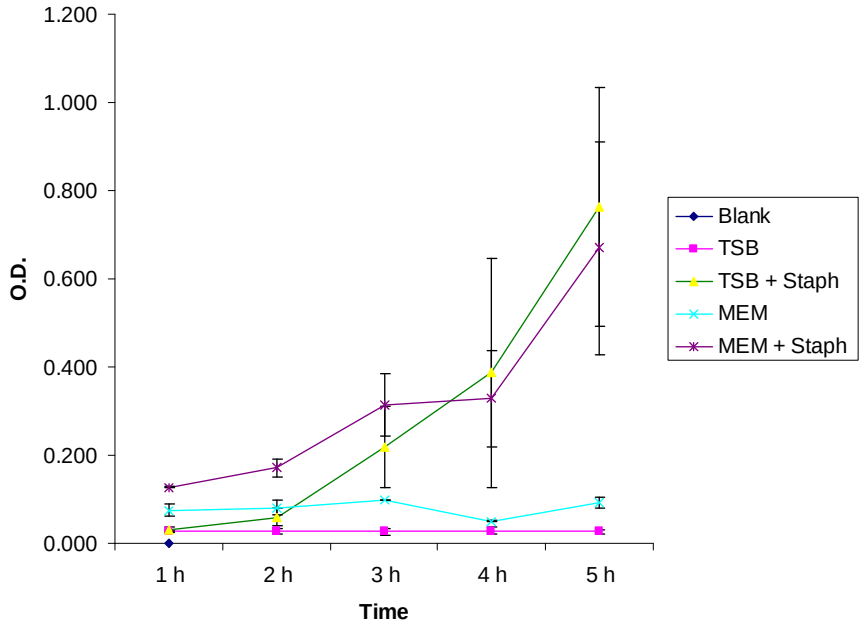


Figure 3.4. Growth curve of *Staphylococcus aureus* in TSB and MEM media.
S. aureus strain Newman was grown overnight in TSB medium or MEM medium rotating at 250 rpm, at 37°C in air. The following day a 1:200 dilution solution was prepared using both media and aliquots of medium were taken hourly to measure the optical density (O.D.). Data are presented as mean ± standard error of the mean (SEM) of four consecutive experiments.

Once I established that the growth of Staphylococci was comparable in cell culture medium and TSB, I went on to design a model to mimic the early stages of colonisation, for which the infecting doses were at a ratio of approximately 1 bacterium per 10 cells (1. x 10^5 colony forming units (cfu)/ml) or approximately 1 bacterium per 6 cells (1.6 x 10^5 cfu/ml). RPMI 2650 cells were cultured in MEM media in the absence of antibiotics and infected with *S. aureus* strain Newman at the infecting doses calculated above. (Figure 3.5)

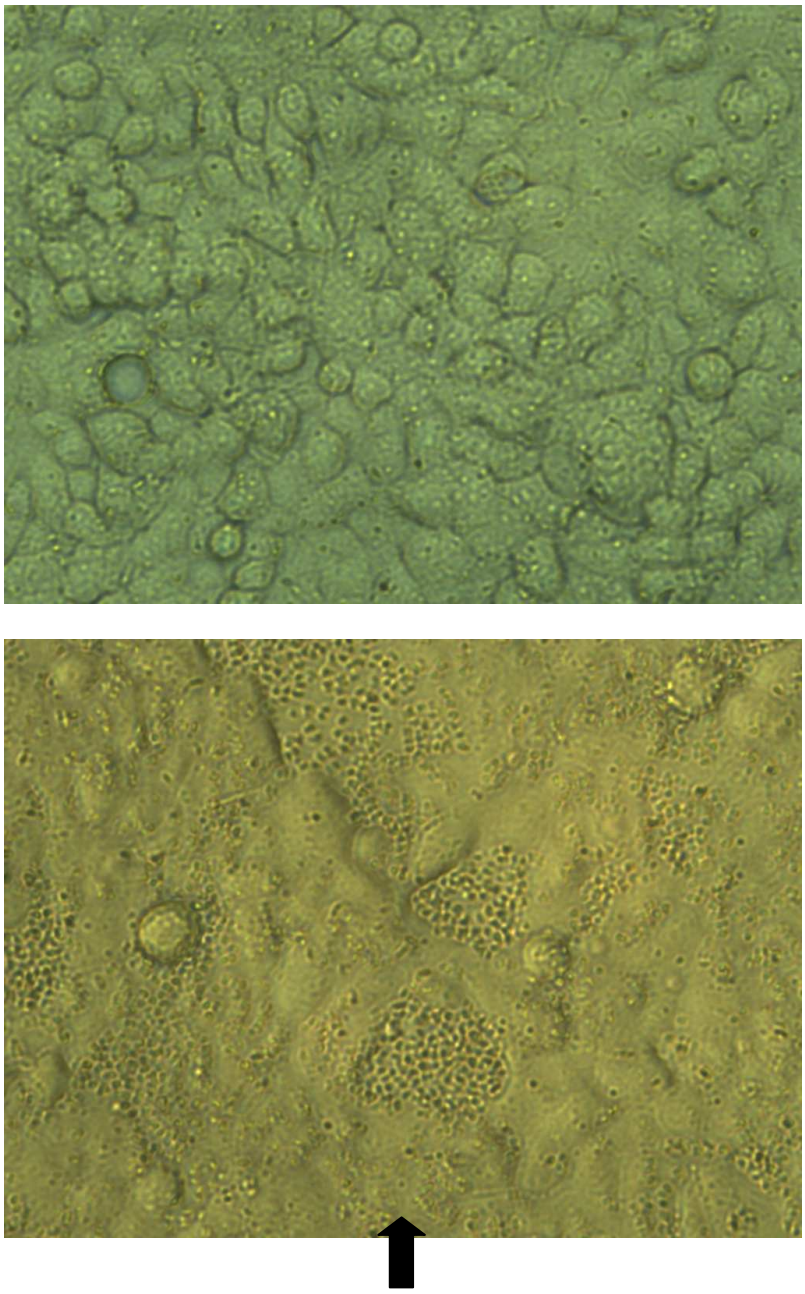


Figure 3.5. Co-culture of *S. aureus* strain Newman with the cell line RPMI 2650.
Cells were plated at 1 x 10^6 cells/ml and infected with 1.1 x 10^5 cfu of *S. aureus*, microphotographs were taken prior to infection a) and at 3 hours (b). Magnification 2000x. Arrow: Staphylococcal microcolony formation.

After infection with *S. aureus* strain Newman, within 2 hours it was possible to observe rapid growth of the bacterium, by three hours there was evidence of microcolony formation by the bacteria within the cell monolayer. The normal cellular architecture was destroyed at 3 hours with loss of the cellular morphology and invasion of the monolayer by the bacterium. Beyond 4 hours the cellular layer was barely discernible.

During these cultures it was possible to observe the rapid proliferation of the bacteria among the eukaryotic cells. These experiments were performed in the absence of antimicrobials in the culture medium in order to provide a physiological environment to model the colonisation status. Establishing how the dynamics of bacterial colonisation happen is difficult, since it is a highly complex process. On one hand, if the infective dose is too high, a severe immune response will be initiated and the pathogen will be cleared. Therefore, I assumed that the infective dose should be small enough to avoid immunological clearance, but at the same time large enough to ensure the survival of the pathogen. Unfortunately, *S. aureus* proved to behave in an aggressive way with the cells, with formation of Staphylococcal micro-colonies within the epithelium at 3 hours, and loss of architecture by 4 hours. Several approaches were attempted in order to delay the onset of cellular death, such as shortening the time points and decreasing the infective dose to a ratio of 1 cfu per 100 cells. Despite this it was not possible to improve the performance of the model.

3.2.1.3.1. Quantitative PCR during *S. aureus* infection

During the work with prokaryotic and eukaryotic cell cultures, quantitative PCR (QPCR) was performed to assess individual eukaryotic gene responses during infection.

The cell line RPMI 2650 was infected as described above and QPCR for the gene of interest and for the housekeeping genes *GAPDH* was performed. It was observed that after a 4 hour infection of RPMI 2650 cells with *S. aureus*, multiple gene changes happened simultaneously, particularly when assessing housekeeping genes. Housekeeping genes are genes that are required for the maintenance of basal cellular function and are constitutively found in all human cells (Eisenberg, E., and Levanon, E., 2003) It is expected that these genes should be transcribed at a constant rate and that they should provide a baseline to establish further comparisons. I expected to find no variation in the quantification of these transcripts. On the contrary, *GAPDH* mRNA transcripts were constitutively expressed at a low level and appeared to be up-regulated after infection. In view of this finding I went onto assess a different housekeeping gene and performed QPCR for the *18S* gene which is involved in protein synthesis and which according to the literature is a commonly gene used for establishing a baseline. Contrary to expectation, in these experiments it was possible to observe that the *18S* gene also displays great variability during infection. The general trend appeared to be in the direction of down-regulation (Figure 3.6).

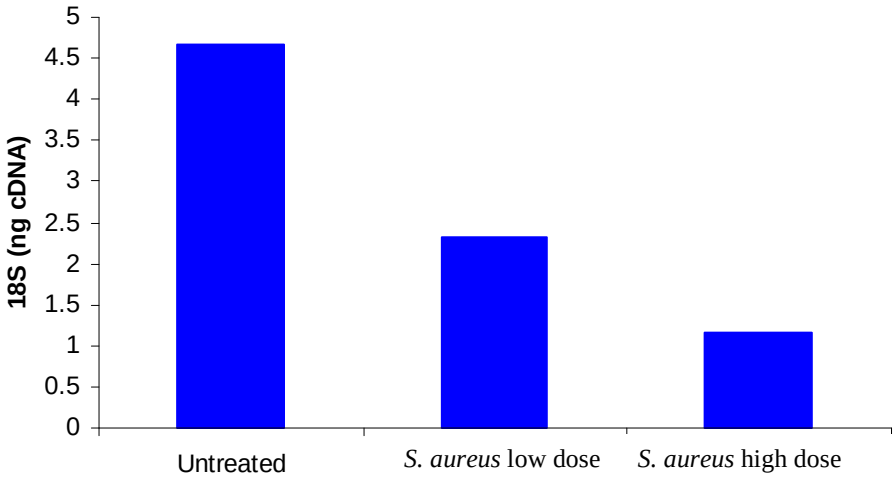


Figure 3.6. Expression of *GAPDH* and *18S* mRNA in the human nasal epithelial cell line RPMI 2650 before and after 4 hours infection with *S. aureus* strain Newman.
RPMI 2650 cells were plated at 1 x 10^6 per well.
Once the cells achieved 90% confluence, they were infected. RNA extraction was performed followed by QPCR using the primers and probes described in section 2.10. Results are presented as a mean of 2 duplicates from one representative experiment.

To my knowledge there is no current evidence in the literature of the effects of *S. aureus* infection on housekeeping genes in human cells. In the case of viral infections, Nystrom, K., et al, 2004 demonstrated that *GAPDH* levels varied by several orders of magnitude during herpes simplex virus type-1 (HSV1) infection of human embryonic lung fibroblasts, although they found less variation in comparison with *18S* mRNA. In addition to this, Dheda, K., et al, 2004, demonstrated a high variability of *GAPDH* in peripheral blood mononuclear cell culture from healthy individuals and patients with tuberculosis. Moreover, Zhong, H., et al, 1999 have reported variability in *GAPDH* among cell types during cell proliferation and development, as well as in response to various stimuli such as hypoxia, insulin, dexamethasone and mitogens as well as to experimental treatments.

The role of *18S* has also been questioned as a housekeeping gene. A study by Araya, M., et al, 2008, demonstrated *18S* as highly unstable when comparing nine candidate housekeeping genes during the study of haemocytes from soft-shell clams challenged with *Vibrio splendidus*. Furthermore, the effects of adenovirus infection on *28S* and *18S* rRNA synthesis and maturation in HeLa cells were described in 1983 by Castiglia, C. and Flint, S. They observed that after adenoviral infection protein synthesis was inhibited rapidly.

The overwhelming effect that the bacterial infection had on the cellular monolayers was documented when real-time PCR was performed in the monolayers. The detection of the genes *GAPDH* and *18S* showed an alteration in

their expression which might be explained by alterations in the consumption of energy and ribosomal protein synthesis as a result of the bacterial infection. The results may indicate that during an *in vitro* bacterial infection, and without an innate immune system that would assist in controlling the infection, the cells are overwhelmed by the injury and multiple cellular processes happen simultaneously, making this model a very difficult one in order to establish mechanisms. In addition, cytokine measurements in supernatants could not be performed by conventional ELISA methods, due to the interference of the Staphylococcal virulence factor protein A, which binds with high avidity the Fc fraction of IgG (Kessler, S., 1975). Prokaryotic and eukaryotic co-cultures were also attempted with the methicillin-resistant *Staphylococcus aureus* (MRSA) strain 252 and with isogenic mutant strains with deletions in *clfb* and *protein A*, with similar results to those previously described. Due to these difficulties it was considered that this was a highly complex model in which it would not be possible to establish single differences involved in the epithelial recognition of, and response to, Staphylococcal antigens, and it was decided to change the approach to using a sterile microenvironment model in which variables could be controlled in a better manner.

The advantage of using a sterile model that mimics exposure to single virulence factors was demonstrated in primary human nasal epithelial cells, where it was shown that bacterial ligands did not change expression of the housekeeping gene

18S. After implementing a sterile method in which cells were stimulated with bacterial ligands such as *Pseudomonas aeruginosa* LPS, *Staphylococcus aureus* PGN and LTA and the pro-inflammatory cytokine TNF-α, *18S* transcripts were measured over a 24 hour period (Figure 3.7). Although baseline levels of *18S* appeared lower in comparison to the baseline levels found in the RPMI 2650 cell line (shown in Figure 3.6), it was noted that they remained constant throughout the 24 hour period and they were not affected by the different experimental conditions.

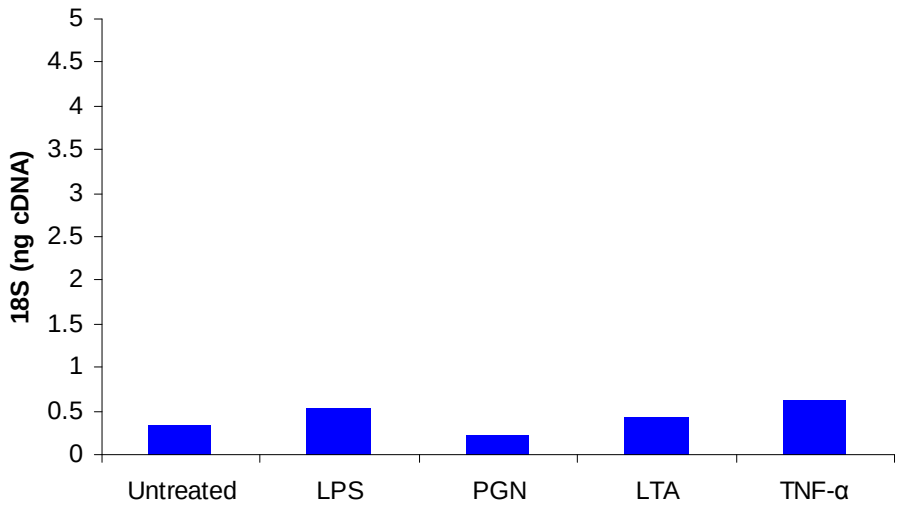


Figure 3.7. Expression of *18S* mRNA in the human primary nasal epithelial cells after 24 hours stimulation with TLR ligands
Primary human nasal epithelial cells were plated onto uncoated plastic plates. Once the cells achieved 90% confluence, they were stimulated with bacterial ligands. RNA extraction was performed followed by QPCR using the primers and probe as described in section 2.10 of Materials and Methods.
Results are presented as a mean of replicates from one representative experiment.

3.2.1.4. Cytokine production by the cell line RPMI 2650 after stimulation with TLR ligands

RPMI 2650 cells were cultured and stimulated with bacterial ligands such as *P. aeruginosa* LPS at 10ng/ml and 100ng/ml, *S. aureus* PGN at 10µg/ml and 100µg/ml, *S. aureus* LTA at 10µg/ml and TNF-α at 10ng/ml for 24 hours. Cell viability was assessed using trypan blue exclusion. Cell viability was not significantly affected by the experimental conditions (Figure 3.8)

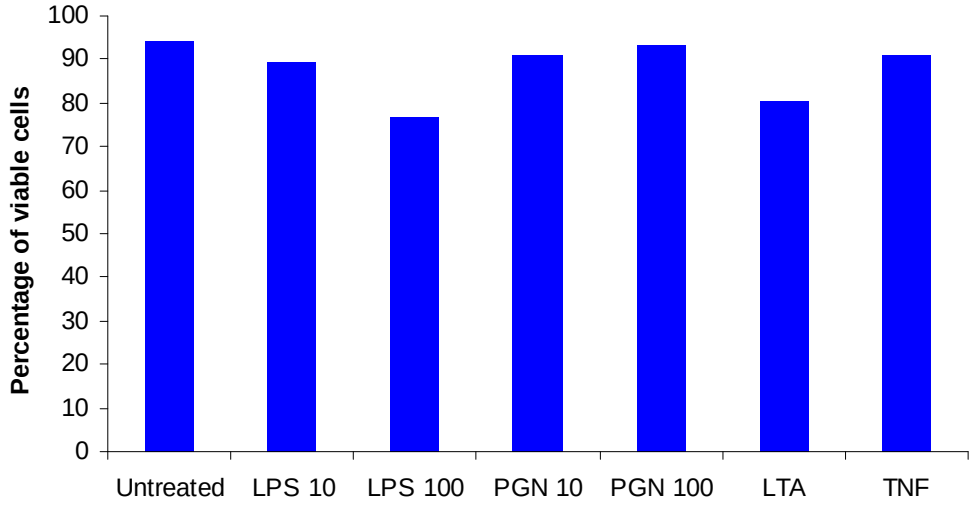


Figure 3.8 Viability counts in RPMI 2650 cells after stimulation with TLR ligands
Cells were seeded in 6 well plates at $2x10^6$ cells/ml and stimulated with bacterial ligands as described above. Cells were trypsinised and viability counts were performed using trypan blue exclusion method.
Data are presented as percentage of viable cells for one representative experiment.

LPS activity was assessed using the Limulus amoebocyte lysate (LAL) assay. *P. aeruginosa* PAO1 LPS activity was comparable in activity to *E. coli* LPS (data courtesy of Dr. Hsin- Ni Li, University of Edinburgh).

RPMI 2650 cells were stimulated with TLR ligands as well as with the pro-inflammatory cytokine TNF-α as described above. Despite of being viable, median constitutive levels of IL-8 and SLPI were non detectable by ELISA in supernatants; neither did these cells respond to stimulation. The secretion of IL-6 was very low; just at the limit of detection of the ELISA assay and it was not affected by stimulation. Constitutive median production of the anti-inflammatory cytokine IL-10 by the cell line RPMI 2650 was 32.8 pg/ml (range: 0-118). This was not increased after incubation with bacterial ligands such as LPS, PGN or LTA, nor by stimulation with the pro-inflammatory cytokine TNF-α.

3.2.2. Characterisation of the primary nasal epithelial cells

3.2.2.1. Description of healthy volunteers and patients

A total of 44 subjects were consented for the isolation of primary nasal epithelial cells. Of these 22 were healthy volunteers (HV) and 22 were patients undergoing partial lobectomy or pneumonectomy in the Department of Cardiothoracic Surgery, Royal Infirmary of Edinburgh. Regarding the HV group; there were 16 males and 6 females, mean age was 31 years (range: 24-42), and there were 3

smokers. Successful cultures were achieved for 12 subjects (54%). Cultures were deemed unsuccessful if the cells failed to attach to the collagen matrix (7 subjects) or if the cells failed to achieve confluence in culture after three weeks (3 subjects).

The patient group consisted of 11 males and 11 females who provided consent for nasal brushings to be performed The mean age of this group was 65 years (range: 53-76), and 17 were smokers. Successful cultures were achieved for 10 subjects (45%). Twelve cultures were unsuccessful: one due to fungal contamination and eleven due to failure of the cells to attach to the collagen matrix (Table 3.1).

Median time to confluence was 1 week for the first passage and subsequently cells were sub-cultured in separate wells for the specific experiments, in which the median time to confluence was 2 weeks. All the experiments were performed with cells at passage 2.

Subject	*Age*	*Sex*	*Smoker*	*Culture Successful*	*Reason for no success*
HV1	42	M	N	Y	
HV2	31	M	N	Y	
HV3	34	M	N	N	Failed to attach
HV4	35	F	N	Y	
HV5	34	M	N	Y	
P1	75	M	Y	N	Fungal infection
P2	53	F	Y	N	Failed to attach
HV8	26	F	N	Y	
P3	66	M	Y	Y	
HV10	33	M	N	Y	
HV11	26	F	N	Y	
HV12	28	M	N	N	Failed to attach
P4	76	M	N	N	Failed to attach
HV14	28	M	N	N	Unable to reach confluence
HV15	30	M	N	N	Failed to attach
HV16	30	M	N	Y	
HV17	28	M	N	Y	

HV18	32	M	N	Y	
HV19	34	M	N	N	Failed to attach
P5	59	F	Y	N	Failed to attach
P6	57	M	Y	Y	
HV22	35	M	N	N	Unable to reach confluence
HV23	33	F	Y	N	Unable to reach confluence
P7	70	F	Y	N	Failed to attach
P8	63	F	N	N	Failed to attach
P9	64	F	Y	Y	
HV27	32	F	Y	N	Unable to reach confluence
HV28	34	M	N	N	Failed to attach
P10	71	M	Y	Y	
HV30	24	M	N	Y	
HV31	32	F	Y	Y	
HV32	36	M	N	N	Failed to attach
P11	59	F	N	N	Failed to attach
P12	60	F	Y	N	Failed to attach
P13	67	M	Y	Y	

P14	77	F	N	N/A	Did not consent
P15	61	M	N	Y	
P16	60	F	N	Y	
P17	69	F	Y	N	Failed to attach
P18	73	F	Y	N	Failed to attach
P19	72	M	N	Y	
P20	57	M	Y	Y	
P21	59	M	Y	N	Failed to attach
P22	71	M	Y	N	Failed to attach
P23	69	F	Y	Y	
Total				**22**	**22**

Table 3.1. Demographic data for healthy volunteers and patients from whom primary nasal epithelial cells were obtained.
HV, Healthy Volunteer; P, Patient; N/A, non applicable.

3.2.2.2. Flow cytometry in primary nasal epithelial cells

In order to establish the phenotype of the cells obtained during nasal brushings, cells from 20 healthy volunteers whom already had consented for nasal brushings were used for flow cytometry. For further details about the phenotype of the cells isolated please refer to Chapter No5, Section 5.1.4, page 223.

3.2.2.3. Morphological appearance of the primary nasal epithelial cells

Immediately after the brushings were performed the cell suspension was plated onto pre-coated type I collagen wells and it was possible to visualise ciliated cells. After the culture was established and reached 90% confluence it was possible to visualise the typical "cobblestone" morphology under light microscopy as described by McDougall, C., et al, 2008. Some cells exhibited slight prolongations (Figure 3.9)

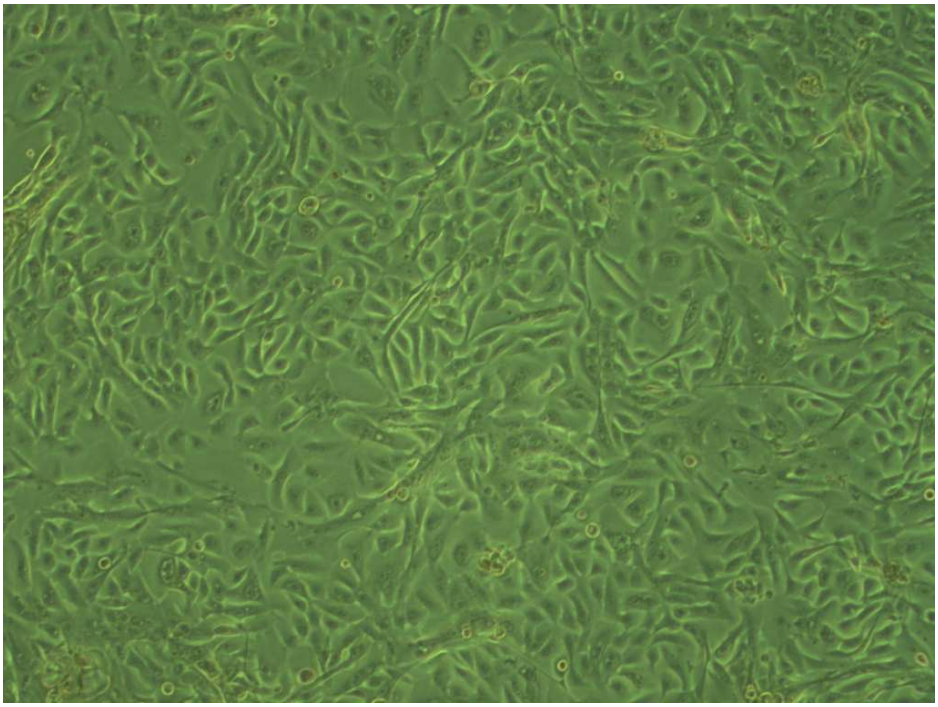
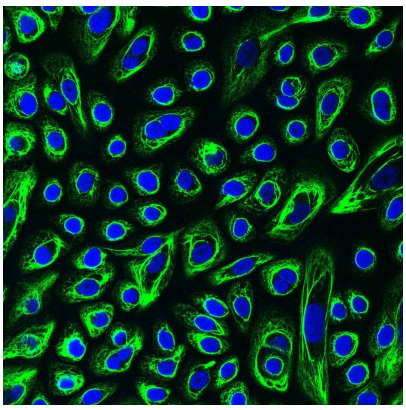


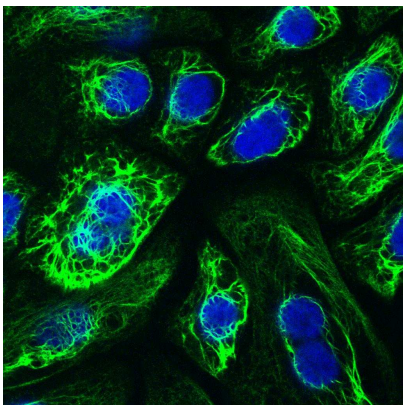
Figure 3.9 Morphology of human primary nasal epithelial cells.
Cells were obtained after brushing of the inferior turbinate of the nose and seeded onto plastic plates pre-coated with type I rat-tail collagen. Cells reached confluence after approximately 7 days. Magnification 650x. Cells display the typical cobblestone appearance, some cells also display a slightly elongated appearance.

3.2.2.4. Phenotyping of primary nasal epithelial cells

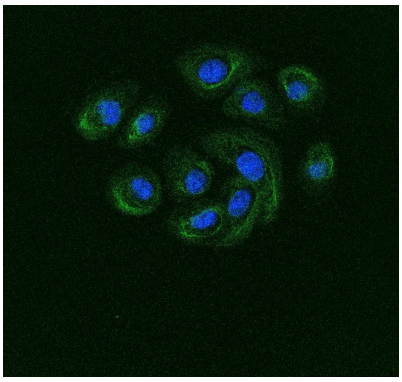
Phenotyping of primary nasal epithelial cells showed a high content of cytokeratin 18 and cytokeratin 19. I was unable to detect cytokeratin 10 expression in these cells. Vimentin staining of the cells was variable - 80% of the cells stained positive for vimentin with 27% of the cells staining strongly to it, whereas all cells (100%) stained positive for the epithelial markers CK18 and CK19. (Figure 3.10)

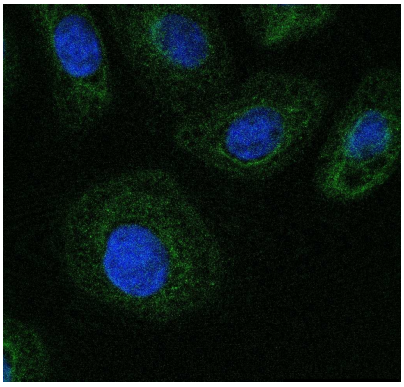
Low magnification High magnification



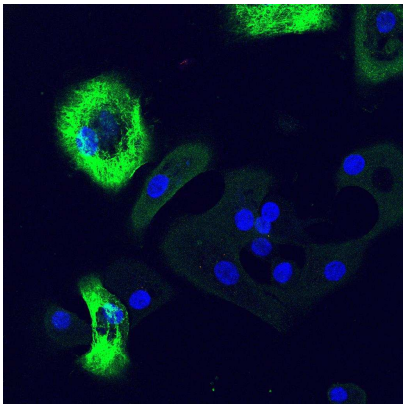


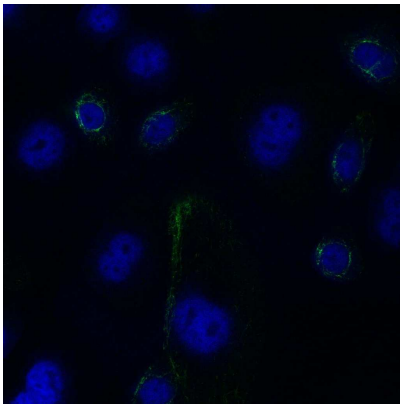
a) CK 18



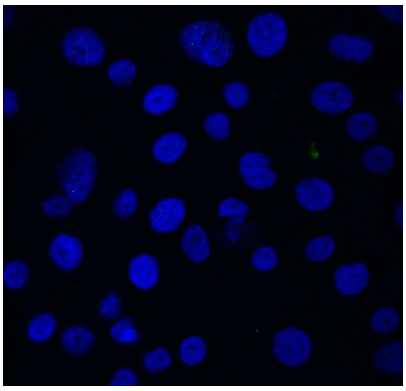


b) CK 19

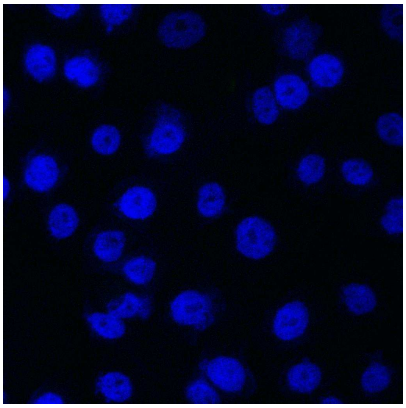




c) Vimentin



d) CK 10



e) Isotype control

Figure 3.10 Phenotyping of human primary nasal epithelial cells.
Primary human nasal epithelial cells were plated on coverslips, fixed with 4% paraformaldehyde solution, blocked with 2% goat serum, and subsequently incubated with murine monoclonal antibodies against a)Cytokeratin 18, b)Cytoketatin 19, c)Vimentin, d)Cytokeratin 10 and e)Isotype control. Nuclei were stained with DAPI (blue). Secondary antibody was anti-mouse IgG linked to Alexa 488(green).
Images were analysed using confocal microscopy. Scale bars: 50μm for low magnification images and 25μm for high magnification. The typical morphology of cytokeratin filaments is observed.

3.2.2.5. Cytokine production by primary nasal epithelial cells

Culture and isolation of primary human nasal epithelial cells was difficult due to problems in obtaining volunteers and because it is a demanding, skilled technique. In addition the attachment and proliferation of cells during culture varies form subject to subject. Cell-free supernatants were removed and assayed by ELISA initially and then standardised using the CBA kit in order to avoid operator error which could occur during ELISA.

The cytokines assayed included IL-8, IL-6, IL-1β, IL-12p70, IL-10 and TNF-α. Within this selective group of cytokines IL-8, IL-6 and IL-1β were chosen as there is evidence that these are secreted in response to stimulation with TLR ligands by respiratory epithelial cells (Cheon, I., et al, 2008; Burvall, K., et al, 2003; Berube, J., et al, 2009). TNF-α and IL-12p70 were chosen as they are predominantly secreted by macrophages, T cells and dendritic cells (particularly the latter one), and in this way it would be possible to demonstrate functionally that the monolayers were predominantly composed by epithelial cells and that there was no contamination with the previously mentioned cell types (Bekeredjian-Ding, E., et al, 2006 and Barkman, C., et al, 2008). Finally, IL-10 was chosen as a cytokine known by its anti-inflammatory properties. Whilst it would have been ideal to report the levels for the cytokine TGF-β2 and the antimicrobial peptide SLPI, it was not possible to produce these results due to lack of supernatant. It would be of great interest to include these molecules in

future research studies. It is also relevant to mention that due to limited numbers of cells obtained during the preparations, the cell lysates were used for performing RNA extraction and PCR, therefore cytokine correction in supernatants to total protein content in cell lysates was not performed. For this analysis, cells from five patients were included (Table 3.2)

Subject	Age	Sex	Smoker
Patient 3	66	M	Y
Patient 6	57	M	Y
Patient 9	64	F	Y
Patient 20	57	M	Y
Patient 23	69	F	Y

Table 3.2 Characteristics of the five patients from which primary nasal epithelial cells were isolated

Primary nasal epithelial cells produced constitutive levels of all of the cytokines assayed (IL-8, IL-6, IL-1β, TNF-α, IL-10 and IL-12p70). There were differences in constitutive production among patient's cells, particularly for IL-8, IL-6 and IL-1β. Stimulation with *S. aureus* PGN and the pro-inflammatory cytokine TNF-α, were associated with an increase in the secretion of the cytokines IL-8 and IL-

6. Stimulation with *P. aeruginosa* LPS or *S. aureus* LTA were not associated with an increase in the levels of IL-8 or IL-6. There was not an associated increase in the secretion of the cytokines IL-1β, TNF-α, IL-10 or IL-12p70 after stimulation with TLR ligands or TNF-α. For further details, please refer to table 3.7, page 143 in which the data are presented.

3.2.3. Characterisation of cells from the airway

3.2.3.1. Primary bronchial epithelial cells

Primary bronchial epithelial cells were obtained using blind brushings through an endotracheal tube as described in the Materials and Methods section. A total of nine sets of primary bronchial epithelial cells were obtained from nine consented patients, of whom only one set was successfully cultured. These cells were used in immunocytochemistry studies (see Chapter 4). One culture was contaminated with a fungal infection and the remaining seven failed to attach to the collagen matrix. Due to the lack of success of this technique it was discontinued after patient number 9 was recruited into the study. It is likely that one of the main limitations in these cultures was that the bronchial brushings were performed blindly. (Table 3.3)

Subject	*Age (Yr)*	*Sex*	*Lobe resected*	*Final diagnosis*	*Smoker*	*Cultures Successful*
1	75	M	RL	Squamous lung cell Ca	Y	N
2	53	F	RL	Aspergilloma	Y	N
3	66	M	LL	Non small cell lung Ca	Y	Y
4	76	M	RL	Single lung metastasis from bowel Ca	N	N
5	59	F	LU	Squamous cell Ca	Y	N
6	57	M	LL	Non small cell lung Ca	Y	N
7	70	F	RU	Bronchogenic Ca	Y	N
8	63	F	L*	Single lung metastasis from malignant Melanoma	N	N
9	64	F	L*	Squamous cell Ca	Y	N

Table 3.3 List of patients from whom primary bronchial epithelial cell cultures were attempted.
Patients who underwent lung resection were consented. After induction of anaesthesia, a bronchoscopy brush was inserted blindly through the endotracheal tube and bronchial brushings were performed as described in the Materials and Methods section. *pneumonectomy specimens

3.3. Characterisation of the cells of the lower respiratory tract

3.3.1. Characterisation of the cell line A549

The cell line A549 has been characterised in the literature (Lieber, M, et al, 1976; Foster, K., et al, 1998) as a type II alveolar epithelial cell model for metabolic studies and also as a model for *in vitro* characterisation of human alveolar epithelial cell functions.

A549 cells have been reported to produce a cobblestone morphology under light microscopy. (Figure 3.11, courtesy of Dr. Tom Wilkinson)

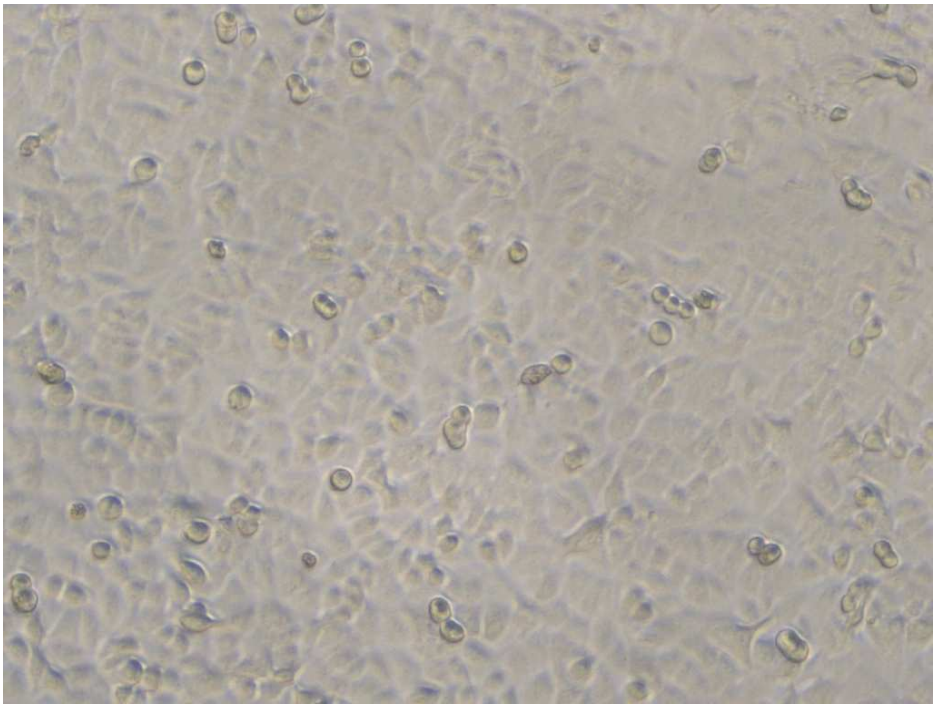


Figure 3.11 Light microscopy of the cell line A549
A549 cells were cultured in DMEM supplemented with L-glutamine and 10% foetal calf serum. Cells were seeded in 6 well plastic Costar plates at 2.5×10^6 per well and allowed to reach confluence. Magnification x 650. Courtesy of Dr. Thomas Wilkinson

3.3.1.1. Constitutive cytokine production

Mean production of TGF-β2 by A549 cells was 50.94 (+44.19) pg/mg of lysate protein in cell free cultured supernatant after four hours of culture and these levels rose to 185.3 (+121.2)pg/mg protein after 24 hours, this increase was statistically significant ($p < 0.05$).(Figure 3.12)

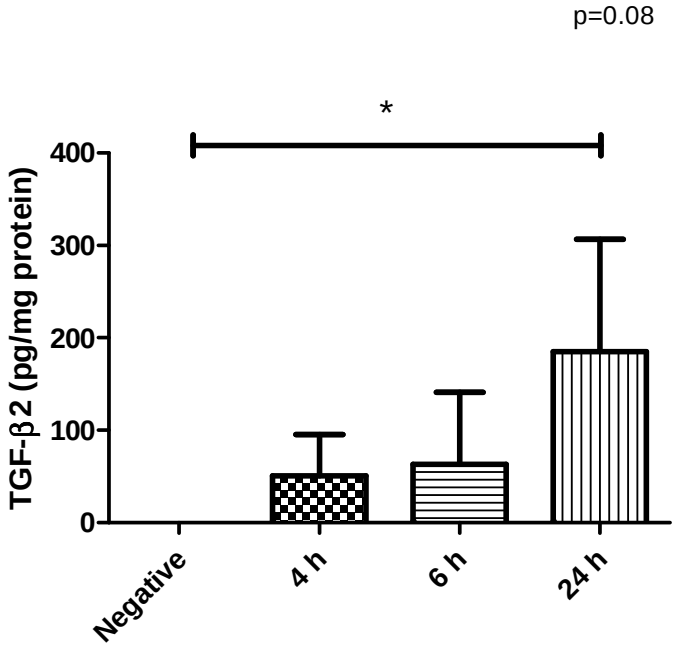


Figure 3.12 TGF-β2 production in supernatants by the human alveolar type II cell line A549 (n=3).
The cells were plated at 5 x10^5 cells/ml in a 24 well plate. Once they achieved 90% confluence, supernatants were removed at different time points and TGF-β2 levels were measured by ELISA and normalised to total protein content in cell lysates. Negative control: cell-free medium
Data are presented as mean (columns) ±SD (error bars) and analysed using ANOVA. Post-test: Dunn's test
Statistical significance (*) determined when $p<0.05$

Burvall, K., et al, 2003 have shown that A549 cells constitutively produce low levels of IL-6 (11 $\pm$ 4 pg per 10^6 cells) and higher levels of IL-8 (167 $\pm$ 25 pg per 10^6 cells).

3.3.1.2. Cytokine production by the cell line A549 after stimulation with TLR ligands

3.3.1.2.1. IL-8

IL-8 levels were measured in cell-free supernatant and normalised to total protein content in the cell lysate. Median constitutive levels of IL-8 were 394.8 pg/mg of lysate protein (range: 205.5 – 560.6). These levels increased significantly after stimulation of the cells with *S. aureus* PGN as well as after stimulation with the pro-inflammatory cytokine TNF-α. The cells remained unresponsive to two different concentrations of *P. aeruginosa* LPS. Stimulation with *S. aureus* LTA did not induce an increase in the median production of IL-8. (Figure 3.13)

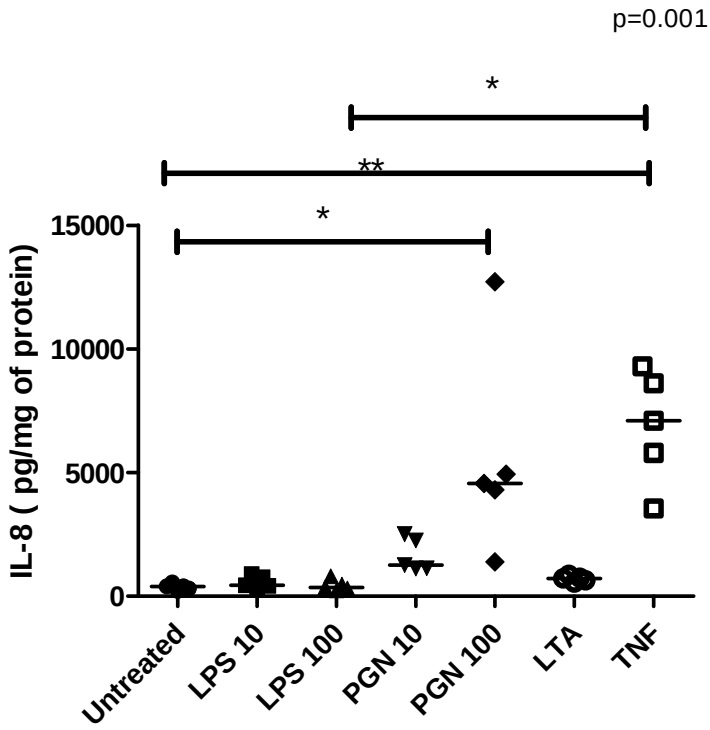


Figure 3.13 IL-8 levels in the A549 cell line after stimulation with TLR ligands (n=5).
RPMI 2650 cells were seeded in 6 well plates at 2 x 10^6 cells/ml. After achieving confluence, cells were washed and stimulated with 10ng/ml and 100ng/ml *P. aeruginosa* LPS, 10μg/ml and 100μg/ml *S. aureus* peptidoglycan, 10μg/ml of *S.aureus* LTA and 10ng/ml of TNF-α for 24 hours. Data are presented as individual data points and median (line) for five consecutive experiments.
Statistical analysis was performed using the Kruskal-Wallis test with Dunn's post hoc analysis using GraphPad Prism software.
Statistical significance was determined when $p<0.05$.

3.3.1.2.2. IL-6

Median constitutive production of IL-6 by the A549 cell line was undetectable. Levels rose significantly after stimulation with *S. aureus* PGN at the higher dose as well as after stimulation with TNF-α. As observed with IL-8, stimulation of

the cells with *P. aeruginosa* LPS or *S. aureus* LTA did not induce an increase of this cytokine.(Figure 3.14)

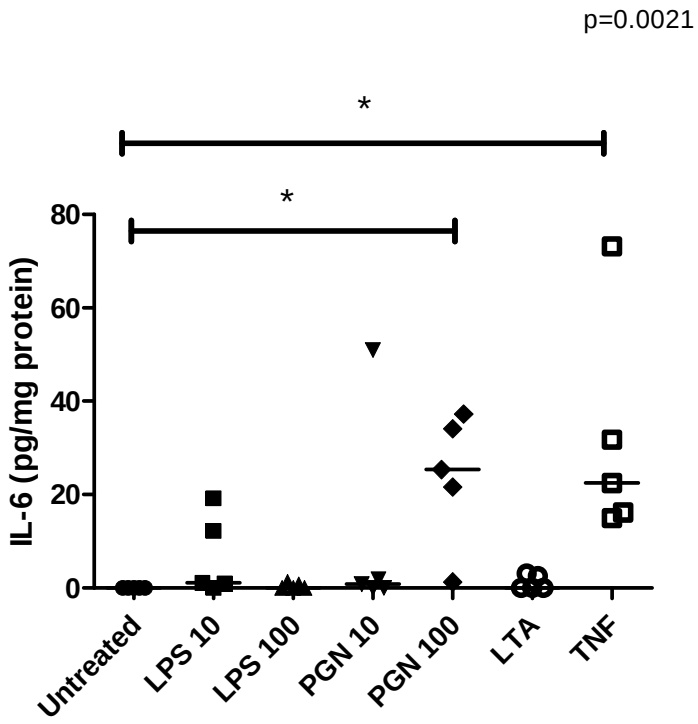


Figure 3.14 IL-6 levels in the cell line A549 after stimulation with TLR ligands (n=5).
A549 cells were seeded in 6 well plates at 2 x 10^6 cells/ml. After achieving confluence, cells were washed and stimulated as described in Figure 3.23.
Data are presented as individual data points and median (line) for five consecutive experiments.
Statistical analysis was performed using the Kruskal-Wallis test with Dunn's post hoc analysis using the GraphPad Prism software.
Statistical significance was determined when $p<0.05$.

Median baseline levels of IL-10 in the A549 cell line were 4.9 pg/mg of protein (range: 0 – 137.8). Median baseline levels of the antimicrobial peptide SLPI in supernatants were 8132 pg/mg of protein (range: 406 – 9423). Stimulation with

TLR ligands such as LPS, PGN and LTA or with TNF-α was not associated with an increase in the secretion of IL-10 or SLPI.

3.3.2. Characterisation of primary cells from the lower respiratory tract

3.3.2.1. Description of patients

Thirteen patients that underwent partial lobectomy or pneumonectomy in the Cardiothoracic Surgery Department at the Royal Infirmary of Edinburgh consented to donate a suitable size of lung tissue for the purposes of this research. In one case, during surgery it was noted that the primary tumour was not suitable for resection. The total number of tissue samples processed therefore corresponds to twelve patients. Due to a small size of the tissue corresponding to patient 14, it was not possible to perform the cytokine production experiments with the cells isolated from this patient.

Four cultures were unsuccessful: two due to infection and a further two due to failure of attachment of the cells onto the collagen matrix.

Patient demographics are listed in Table 3.4

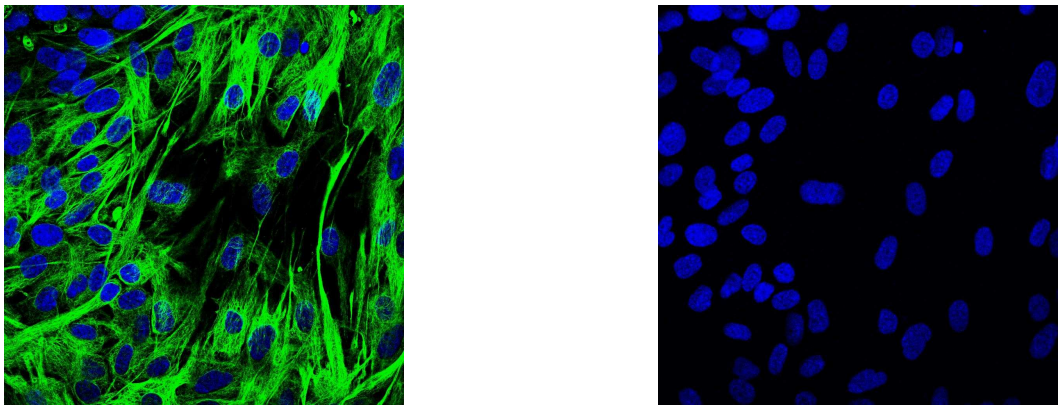
Subject	*Age (Yr)*	*Sex*	*Lobe resected*	*Final diagnosis*	*Smoker*	*Cultures Successful*
1	75	M	RL	Squamous lung cell Ca	Y	N
2	53	F	RL	Aspergilloma	Y	N
3	66	M	LL	Non small cell lung Ca	Y	N
4	76	M	RL	Single lung metastasis from bowel Ca	N	N
5	59	F	LU	Squamous cell Ca	Y	Y
6	57	M	LL	Non small cell lung Ca	Y	Y
7	70	F	RU	Bronchogenic Ca	Y	Y
8	63	F	L*	Single lung metastasis from malignant Melanoma	N	Y
9	64	F	L*	Squamous cell Ca	Y	Y
10	71	M	RU	Squamous cell Ca Lung tissue not obtained-unresectable	Y	N/A
11	59	F	RU	Single lung metastasis from rectal Ca	N	Y
12	60	F	M	Non small cell lung Ca	Y	Y
14	77	F	RL	Lung Adenocarcinoma	N	Y

Table 3.4 Demographics of patients consented for the isolation of primary type II alveolar epithelial cells.
RL, right lower lobe; LL, left lower lobe; RU, right upper lobe; M, right middle lobe; N/A, non applicable; *pneumonectomy

3.3.2.2. Cell types isolated from human lungs

3.3.2.2.1. Primary human fibroblasts

The lung parenchyma has multiple cell types including type I alveolar epithelial cells, primary alveolar macrophages, and Clara cells among many others. Another cell type that was isolated in pure culture was primary human lung fibroblasts which are shown here to illustrate their differences in comparison to primary type II alveolar epithelial cells. On light microscopy they displayed the typical filamentous appearance and on immunocytochemistry they stained positively for vimentin. (Figure 3.15)



a) Vimentin b) Isotype control

Figure 3.15 Primary human lung fibroblasts.
Cells were seeded onto coverslips, fixed with 4% paraformaldehyde, blocked with 2% goat serum and incubated with monoclonal antibodies against a) vimentin or b) with Isotype control. Nuclei were stained with DAPI (blue). Secondary antibody was anti-mouse IgG conjugated with Alexa 488 (green). Images were analysed using confocal microscopy. Scale bar equals 25μm. The typical filamentous morphology of the fibroblasts is observed.

3.3.2.2.2. Primary type II alveolar epithelial cells

3.3.2.2.2.1. Morphology

After the isolation of type II alveolar epithelial cells, the cells were plated onto wells pre-coated with type I bovine collagen and washed several times. Around approximately day 3 - 4 it was possible to observe a confluent monolayer of cells of cuboidal shape (Figure 3.16), certainly quite different from their carcinoma counterpart A549 (Figure 3.11, page 116).

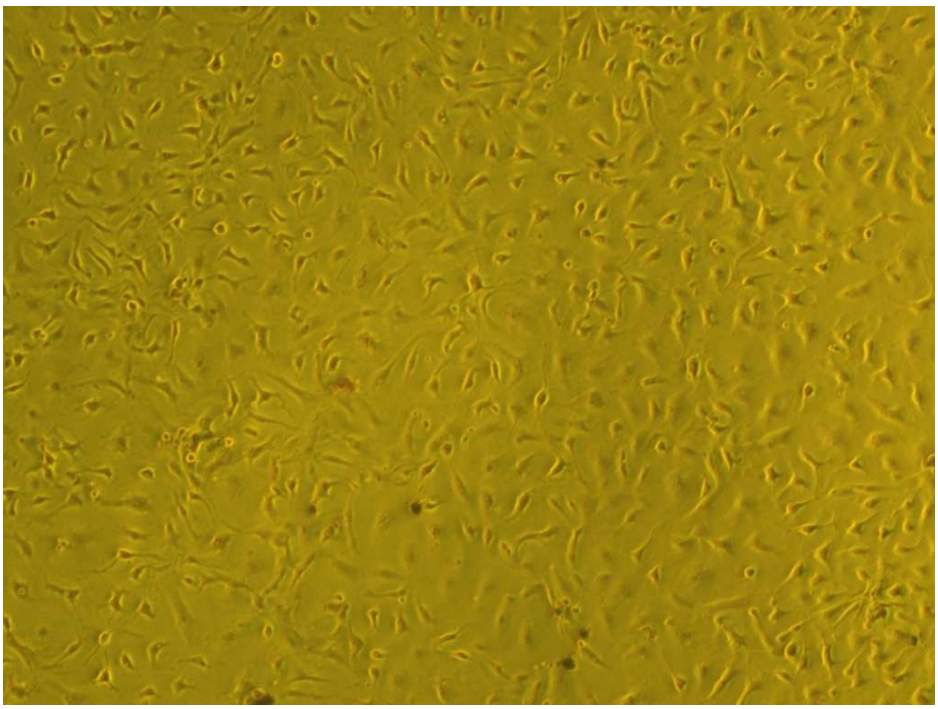


Figure 3.16 Primary human type II alveolar epithelial cells.
Cells were isolated and seeded onto pre-coated type I collagen plates. Cells were washed with HBSS and allowed to reach confluence around day 4. Magnification 650x.

3.3.2.2.2.2. Phenotyping

3.3.2.2.2.2.1. Alkaline phosphatase stain

Alkaline phosphatase has been proposed as a maker of alveolar type II cell differentiation by Edelson et al (1988). This enzyme is not expressed by alveolar macrophages, therefore its histochemical demonstration allows differentiation between type II alveolar epithelial cells and alveolar macrophages.

After the cells achieved confluence around day 3 - 4 alkaline phosphatase staining was performed. The cells took the stain and developed an intense pink colour typical of the type II alveolar epithelial cell phenotype as described by Witherden, I., et al, 2004. (Figure 3.17)

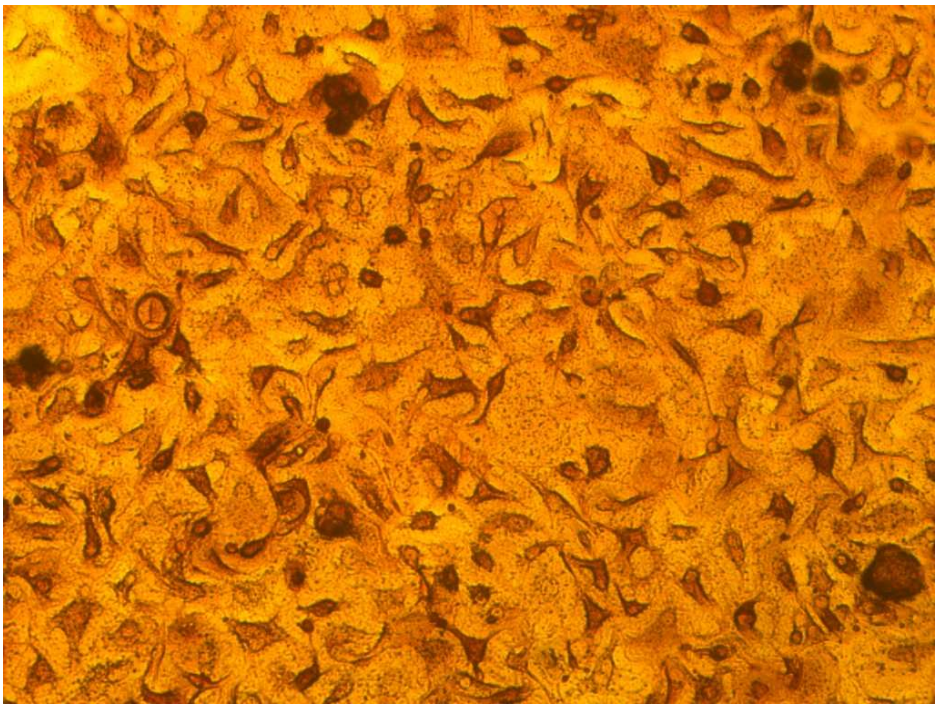


Figure 3.17 Alkaline phosphatase stain for primary human type II alveolar epithelial cells.
Primary type II alveolar epithelial cells were seeded onto plates pre-coated with type I collagen and allowed to reach confluence.
After four days they were stained for alkaline phosphatase and incubated for 15 minutes at 37^{o}C. Magnification 2000x.

3.3.2.2.2.2.2. Rt-PCR for surfactant Protein-C and Aquaporin-3

After demonstrating that the primary type II alveolar monolayers stained positive for alkaline phosphatase, I went onto assess if type II markers such as surfactant protein-C (SP-C) and aquaporin 3 (AQP3) were found in these cells as described by Witherden, I., et al, 2004 and Armstrong, L., et al, 2004.

After the primary type II alveolar epithelial cells achieved a confluent monolayer, they were lysed and RNA was extracted as described in the Materials and Methods section. RT-PCR was performed for SP-C and AQP-3. The type II alveolar epithelial cells displayed m-RNA transcripts. The alveolar type II A549 cell line was used as a negative control and there were no transcripts for these genes found. This finding indicates that the cells displayed primary type II alveolar cell markers. Transcripts of *GAPDH* were used as the housekeeping gene. (Figure 3.18)

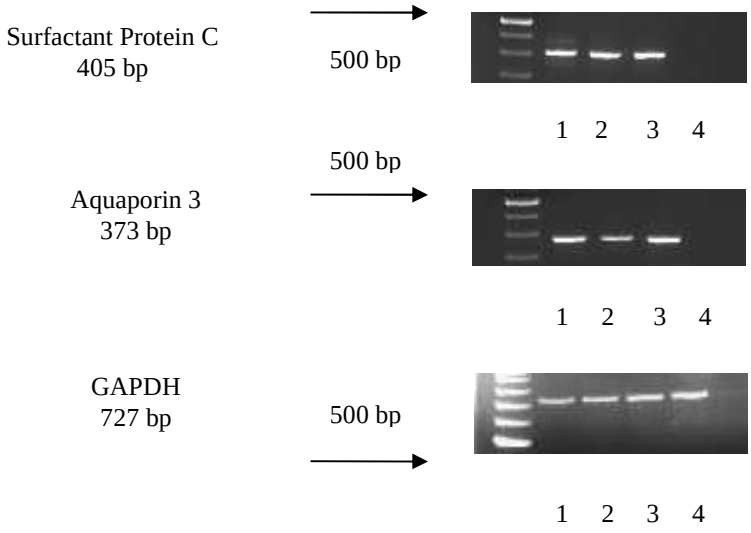


Figure 3.18 Phenotyping of primary human type II alveolar epithelial cells.
Cells were isolated and seeded onto plates pre-coated with type I collagen,. Once confluent, cells were harvested, lysed, RNA was extracted and rt-PCR was performed for SP-C, AQP3 and GAPDH.
Bands labelled as 1, 2 and 3 correspond to patient samples, 4 corresponds to the type II alveolar epithelial cell line A549.

3.3.2.2.2.3. Cytokine response by primary type II alveolar epithelial cells

After type II alveolar epithelial cells achieved a confluent monolayer, they were stimulated with bacterial ligands for 24 hours. Cell supernatants were collected and stored at -80^{o}C until processed, and cells were lysed for RNA extraction, therefore, it was not possible to perform a correction of the total cytokine concentration in supernatant to the total protein concentration in cell lysates for these experiments. Cytokine measurements were performed using the Becton Dickinson (BD) Cytometric Bead Array Human Inflammation kit as described in the Materials and Methods section.

3.3.2.2.2.3.1. Interleukin-8 (IL-8) production

Median basal production of IL-8 by primary type II alveolar epithelial cell was 2273 pg/ml (range: 708 – 11226). This was significantly increased after stimulation with *S. aureus* PGN and the pro-inflammatory cytokine TNF-α. Stimulation with *P. aeruginosa* LPS or with *S. aureus* LTA did not produce an increase in the production of IL-8. (Figure 3.19)

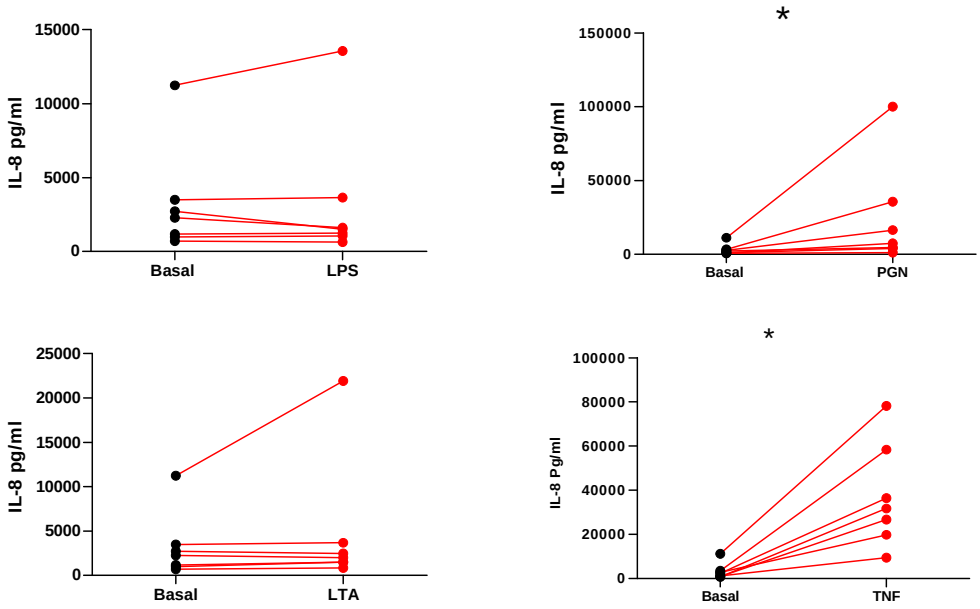


Figure 3.19 IL-8 levels in primary human type II alveolar epithelial cells after stimulation (n=7).
Cells were seeded in 6 well plates pre-coated with type I collagen. After confluence, cells were washed and stimulated with 100ng/ml *Pseudomonas aeruginosa* LPS, 10µg/ml *S. aureus* PGN, 10µg/ml *S. aureus* LTA and 10ng/ml TNF-α for 24 hours.
Data are presented as individual points.
Statistical analysis was performed using the Wilcoxon matched pairs test to compare results for paired samples before and after stimulation using GraphPad Prism software.
Statistical significance (*) was determined when $p<0.05$.

3.3.2.2.2.3.2. Interleukin-6 (IL-6) production

Median basal levels of Interleukin-6 were 236.3pg/ml (range: 8.3 – 1276). The production of this cytokine increased significantly after stimulation with *S. aureus* PGN and the pro-inflammatory cytokine TNF-α. Stimulation with LPS from *P. aeruginosa* or *S. aureus* LTA did not induce an increase in the production of IL-6. (Figure 3.20)

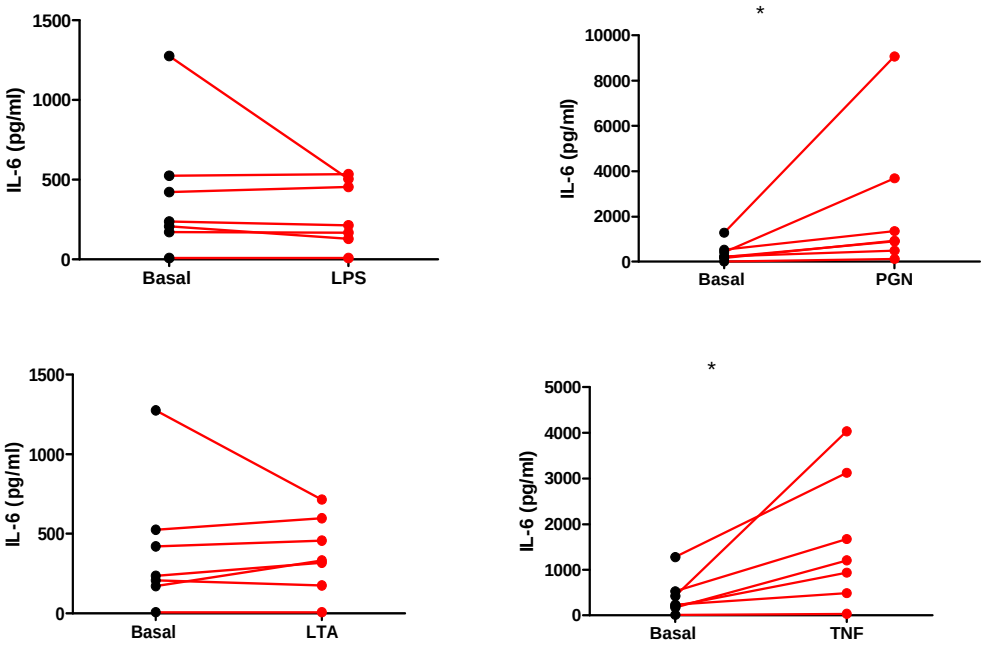


Figure 3.20 IL-6 levels in primary human type II alveolar epithelial cells after stimulation (n=7).
Cells were seeded in 6 well plates pre-coated with type I collagen. After confluence, cells were washed and stimulated in a similar way to Figure 3.31.
Data are presented as individual points.
Statistical analysis was performed using the Wilcoxon matched pairs test to compare results for paired samples before and after stimulation using GraphPad Prism software.
Statistical significance (*) was determined when $p<0.05$.

3.3.2.2.2.3.3. Interleukin-1β (IL-1β) production

Median basal IL-1β production by primary type II alveolar epithelial cells was 5.3pg/ml (range: 2.5 – 8.1). This increased significantly after stimulation with *S. aureus* PGN ($p<0.05$). There was no significant increase associated with stimulation by *P. aeruginosa* LPS, *S. aureus* LTA, or TNF-α. (Figure 3.21)

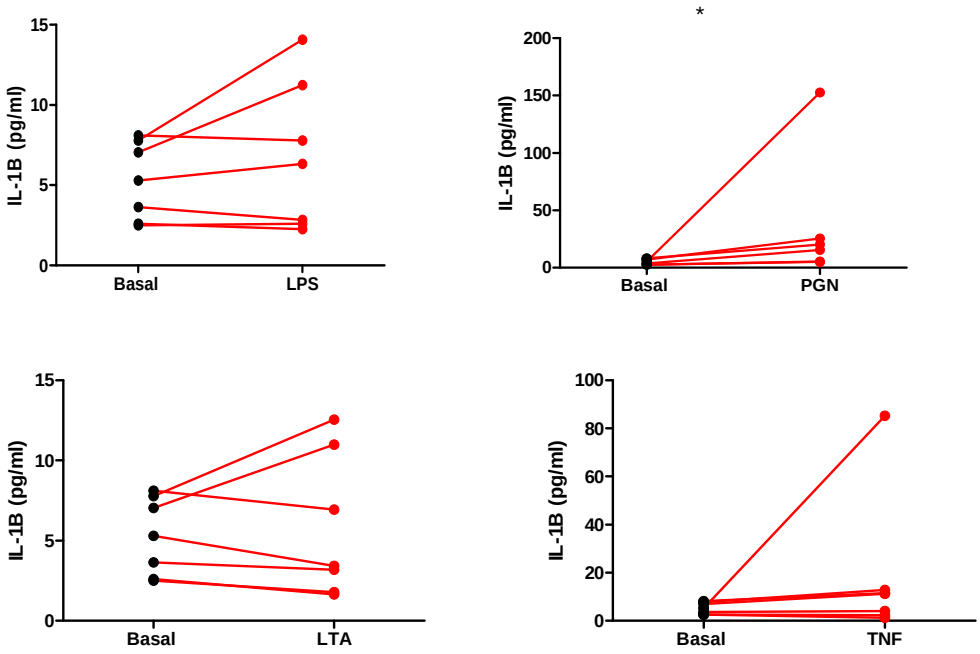


Figure 3.21 IL-1beta levels in primary human type II alveolar epithelial cells after stimulation (n=7).
Cells were seeded in 6 well plates pre-coated with type I collagen. After confluence, cells were washed and stimulated in a similar way as in Figure 3.31. Data are presented as individual points.
Statistical analysis was performed using the Wilcoxon matched pairs test to compare results for paired samples before and after stimulation using GraphPad Prism software.
Statistical significance (*) was determined when $p<0.05$.

3.3.2.2.2.3.4. Tumour Necrosis Factor-Alpha (TNF-α) production

Median baseline production of TNF-α by primary type II alveolar epithelial cells was 10.06pg/ml (range: 3.6 – 21.2). This was significantly increased after stimulation with *S. aureus* PGN. Stimulation with *P. aeruginosa* LPS or *S. aureus* LTA did not show an increase in the production of TNF-α. (Figure 3.22)

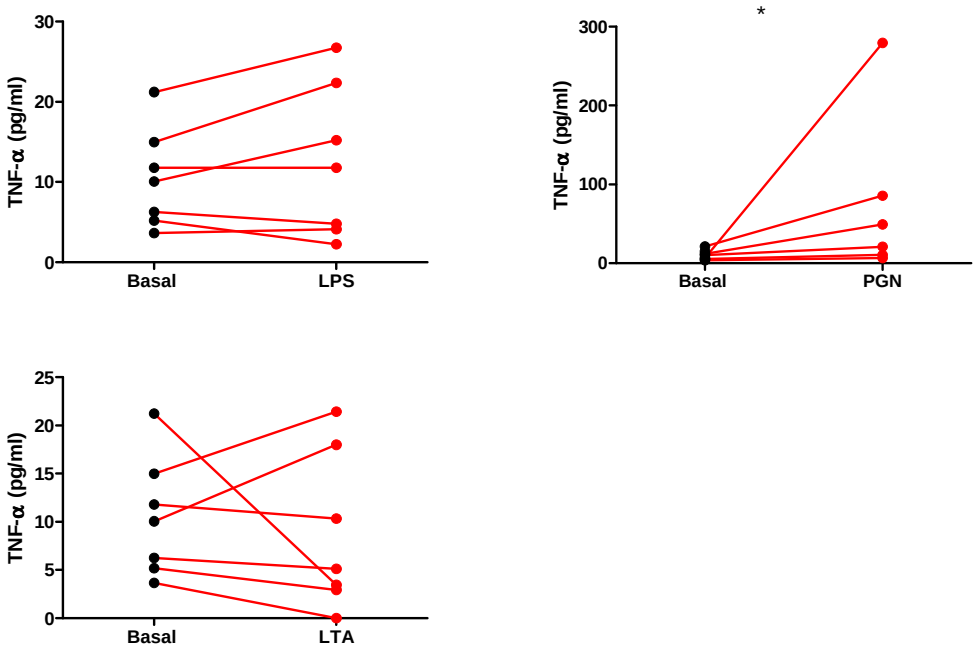


Figure 3.22 TNF-α levels in primary human type II alveolar epithelial cells after stimulation (n=7).
Cells were seeded in 6 well plates pre-coated with type I collagen. After confluence, cells were washed and stimulated in a similar way as in Figure 3.31. Data are presented as individual points.
Statistical analysis was performed using the Wilcoxon matched pairs test to compare results for paired samples before and after stimulation using GraphPad Prism software.
Statistical significance (*) was determined when $p<0.05$.

3.3.2.2.2.3.5. Interleukin-10 (IL-10) production

Median baseline levels of IL-10 produced by type II alveolar epithelial cells were 15pg/ml (range: 2.6 – 1276). A high degree of variability among individuals was noted, with two having particularly high basal secretion of this cytokine. IL-10 levels increased significantly after exposure of the cells to *S. aureus* PGN ($p<0.05$) and TNF-α ($p<0.05$). There was no increase in the production of IL-10 after stimulation with *P. aeruginosa* LPS, or LTA from *S. aureus* (Figure 3.23).

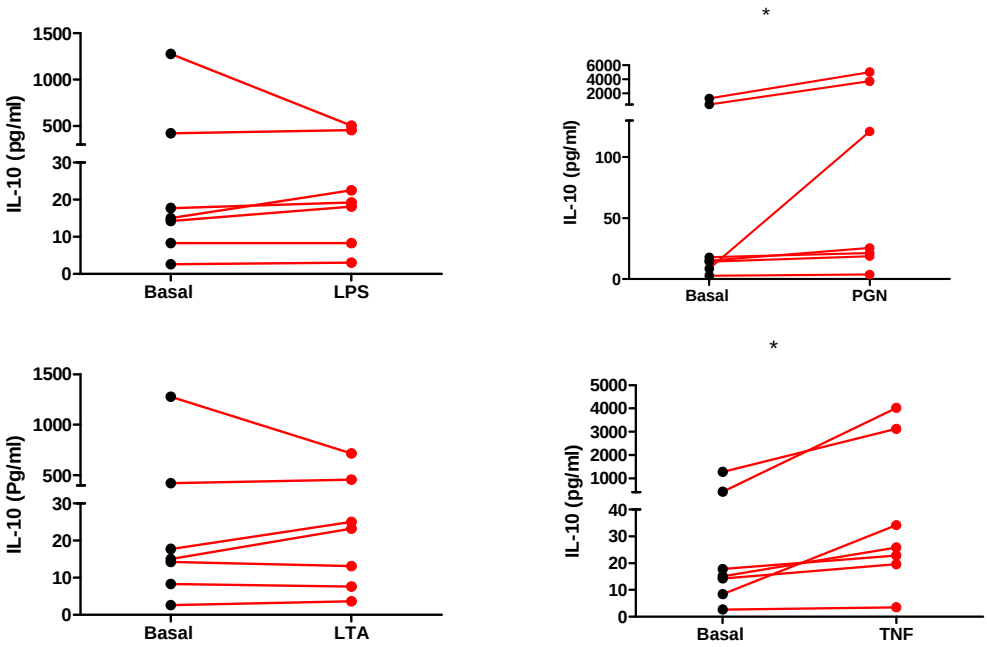


Figure 3.23 IL-10 levels in primary human type II alveolar epithelial cells after stimulation (n=7).
Cells were seeded in 6 well plates pre-coated with type I collagen. After confluence, cells were washed and stimulated in a similar manner as per Figure 3.31.
Data are presented as individual points.
Statistical analysis was performed using the Wilcoxon matched pairs test to compare results for paired samples before and after stimulation using GraphPad Prism software.
Statistical significance (*) was determined when $p<0.05$.

3.3.2.2.2.3.6. Interleukin-12p70 (IL-12p70) production

The median baseline production of IL-12p70 by primary type II alveolar epithelial cells was 8pg/ml (range: 5.4 – 19.7). Despite stimulation with bacterial ligands and the pro-inflammatory cytokine TNF-α, there was not an increase in the production of this cytokine.

3.4. Comparison of the cytokine response between cell lines and human primary cells of the respiratory tract

As it is important to establish the differences in behaviour between cell lines and primary cells, my aim was to compare it using cytokine production as one of the outcomes. Due to the small numbers of primary cells obtained from patients, the following results display total cytokine concentration in supernatants, without protein correction in the lysates as the primary cell lysates were used for RNA extraction. All experiments were conducted in identical conditions.

3.4.1. The cell line RPMI 2650 and primary human nasal epithelial cells

Primary human nasal epithelial cells behave in a different manner to the cell line RPMI 2650. They grow forming a neat monolayer (see figure 3.9, page 108) contrary to the irregular pattern of growth that the cell line displays. Although

they share similar cytokeratin patterns, primary human nasal epithelial cells do not display the presence of cytokeratin 10. Cytokeratin 18 is strongly expressed by both cell types and vimentin expression appears to be stronger in the RPMI 2650 cell line. Primary nasal epithelial cells display variable amounts of vimentin.

The RPMI 2650 cell line was not able to produce actively the pro-inflammatory cytokines IL-8 and IL-6 before or after stimulation with the cytokine TNF-α. On the contrary, primary nasal epithelial cells constitutively secreted these cytokines and responded actively to TNF-α stimulation. RPMI 2650 cells produced higher constitutive levels of IL-10 in comparison to primary nasal epithelial cells and this production was increased after stimulation with TNF-α although not in a statistically significant manner. (Table 3.5)

Cytokine	RPMI 2650 Basal (pg/ml) n=5	RPMI 2650 TNF-α (pg/ml) n=5	p value	Primary nasal epithelial Basal (pg/ml) n=5	Primary nasal epithelial TNF-α (pg/ml) n=5	p value
IL-8	0 (0-50.2)	0 (0)	1	282 (192 – 1004)	2975 (2033-48688)	0.0625
IL-6	6.1(0-16.2)	0 (0-8.4)	0.125	26.2 (13.7 – 167)	452 (67.5 – 3173)	0.0625
IL-10	32.8(0-118)	83(49.6-154)	0.125	10.4(4 - 18.7)	18 (0-67.5	0.3125

Table 3.5 Comparison of cytokine production by RPMI 2650 cells and primary nasal epithelial cells
Statistical analysis was performed using the Wilcoxon matched pairs test to compare results for paired samples before and after stimulation using GraphPad Prism software. Statistical significance (*) was determined when $p<0.05$.

3.4.2. The cell line A549 and primary human type II alveolar epithelial cells

3.4.2.1. Interleukin-8 (IL-8)

Median constitutive production of IL-8 by A549 cells was 765pg/ml. These cells responded to TNF-α stimulation by increasing their IL-8 release. Constitutive median IL-8 production by primary type II alveolar epithelial cells was 2273pg/ml. These cells responded to stimulation with TNF-α by increasing significantly the secretion of this cytokine.(Figure 3.24)

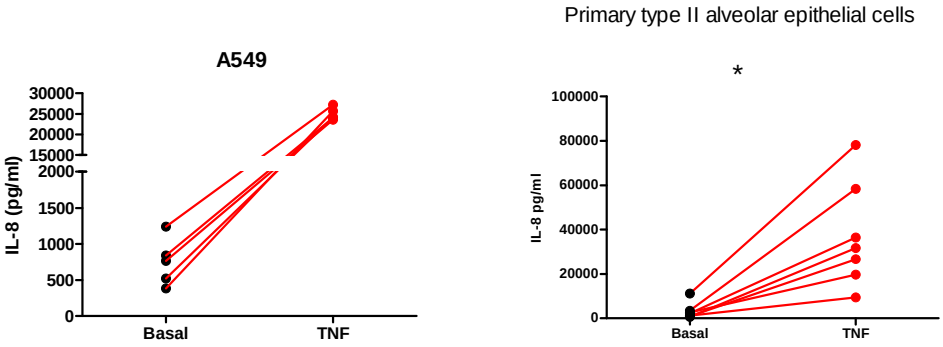


Figure 3.24 IL-8 production by A549 cells (n=5) and primary type II alveolar epithelial cells (n=7).
A549 cells were plated at $2x10^6$ in 6 well plates. After achieving confluence, cells were washed and stimulated with 10ng/ml TNF-α for 24 hours. Primary nasal epithelial cells were treated in a similar way. Cell free supernatants were collected and processed.
Data are presented as individual data points for n=5 for A549 graph and n=7 for primary type II alveolar epithelial cells.
Statistical analysis was performed using the Wilcoxon matched pairs test using GraphPad Prism software.
Statistical significance (*) was determined when $p<0.05$.

3.4.2.2. Interleukin-6 (IL-6)

Median constitutive production of IL-6 from A549 cells was undetectable, although cells responded to stimulation with TNF-α by increasing the production of this cytokine. In primary type II alveolar epithelial cells, median basal levels of IL-6 were 236pg/ml and this increased significantly after stimulation with TNF-α. (Figure 3.25)

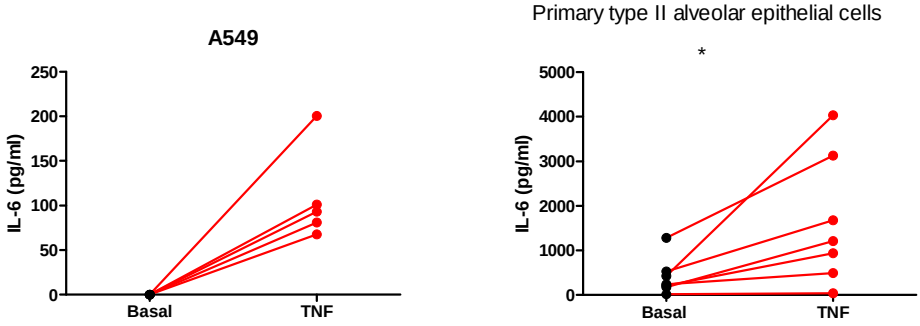


Figure 3.25 IL-6 production by A549 cells (n=5) and primary type II alveolar epithelial cells (n=7).
A549 cells were plated at 2x106 in 6 well plates. After achieving confluence, cells were washed and stimulated in a similar manner as per Figure 3.40.
Data are presented as individual data points for n=5 in A549 graph and n=7 in primary type II alveolar epithelial cells graph.
Statistical analysis was performed using the Wilcoxon matched pairs test using GraphPad Prism software.
Statistical significance (*) was determined when $p<0.05$.

3.4.2.3. Interleukin-10 (IL-10)

Median basal production of IL-10 by A549 cells was 8.4pg/ml. Stimulation with TNF-α was associated with variable results. In two experiments there was a decrease in the secretion of Il-10, whereas in three it was raised. On the contrary, median constitutive IL-10 production by primary type II alveolar epithelial cells was 15pg/ml. This was increased after TNF-α stimulation. It is also important to mention that two subjects produced high baseline levels of this cytokine, which then responded with an increase in the secretion of IL-10 in the medium. (Figure 3.26)

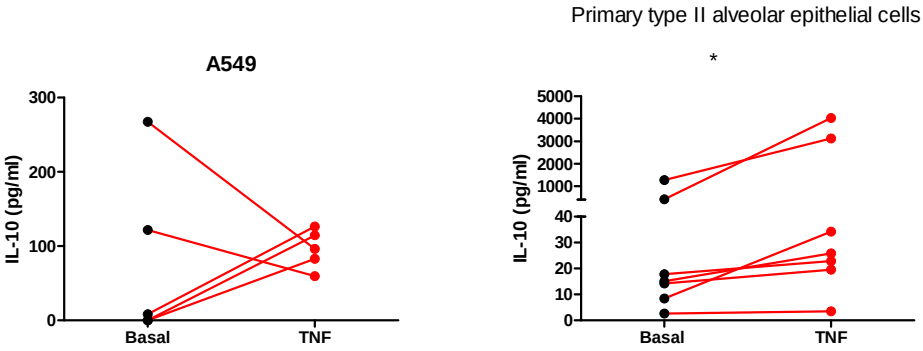


Figure 3.26 IL-10 production by A549 cells (n=5) and primary type II alveolar epithelial cells (n=7).
A549 cells were plated at $2x10^6$ in 6 well plates. After achieving confluence cells were washed and treated in a similar manner as per Figure 3.40.
Data are presented as individual data points for n=5 in the A549 graph and n=7 in the primary type II alveolar epithelial cells graph.
Statistical analysis was performed using the Wilcoxon matched pairs test using GraphPad Prism software.
Statistical significance (*) was determined when $p<0.05$.

Cytokine	A549 Basal (pg/ml) n=5	A549 TNF-α (pg/ml) n=5	p value	Primary type II alveolar epithelial Basal (pg/ml) n=7	Primary type II alveolar epithelial TNF-α (pg/ml) n=7	p value
IL-8	765(385 - 1240)	24192(23562 -27192)	0.0625	2273 (707-11226)	31721 (9450-78198)	*0.0156
IL-6	0	93(67.5-200)	0.0625	236(8.3-1276)	1205(34.1-4029)	*0.0156
IL-10	8.4(0-267)	96(59.6-126)	0.8125	15(2.6-1276)	25.8(3.5-4029)	*0.0156

Table 3.6 Comparison of cytokine response between the A549 cell line and human primary type II alveolar epithelial cells
Statistical analysis was performed using the Wilcoxon matched pairs test to compare results for paired samples before and after stimulation using GraphPad Prism software. Statistical significance (*) was determined when $p<0.05$.

3.5. Comparison between primary nasal epithelial cells and type II alveolar epithelial cells in response to TLR ligands

It is interesting that in clinical practice nasal epithelium favours the presence of micro-organisms without developing an overt inflammatory response, whereas in the lower respiratory tract, and especially in the lung parenchyma, the presence of the same organism is associated with a severe inflammation that if not treated appropriately could even risk the life of the host. In order to answer this question, I have isolated, cultured and performed experiments with primary human epithelial cells from the upper respiratory tract and the lower respiratory tract and their cytokine responses have been analysed in order to gain some insight into this process.

For these experiments, primary nasal epithelial cells were obtained from 5 patients with neoplastic lung pathology and they were matched to 7 patients who underwent partial lobectomy or pneumonectomy as described in the Materials and Methods section 2.2.1 page 59 and section 2.2.3 page 62 respectively.

3.5.1. Interleukin-8 (IL-8)

Median constitutive levels of IL-8 in primary nasal epithelial cells were 282pg/ml, while primary type II alveolar epithelial cells produced higher levels: 2273pg/ml. Both cell types responded by increasing the production of this cytokine after stimulation with TNF-α, although in the alveolar epithelial cells the increase was statistically significant.(Figure 3.27)

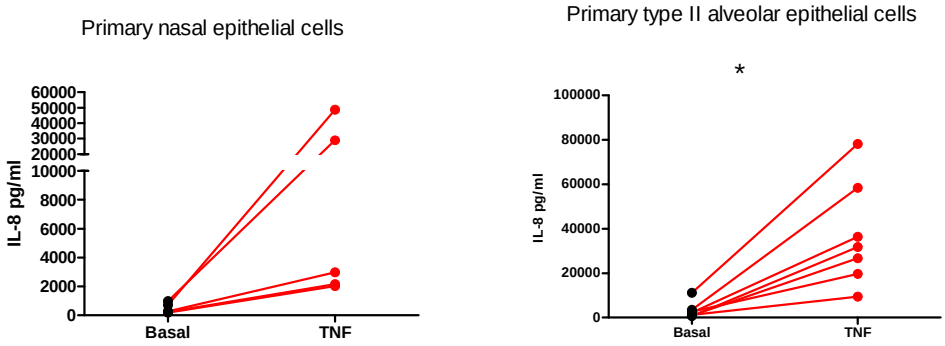


Figure 3.27 IL-8 production by primary nasal epithelial cells and primary type II alveolar epithelial cells.
Primary nasal epithelial cells were plated at 2x106 in 6 well plates, after achieving confluence. Cells were washed and stimulated with 10ng/ml TNF-α for 24 hours. Primary type II alveolar epithelial cells were treated in a similar way. Cell free supernatants were collected and processed by CBA.
Data are presented as individual data points for n=5 in the nasal epithelial cells graph and n=7 in the primary type II alveolar epithelial cells graph.
Statistical analysis was performed using the Wilcoxon matched pairs test using GraphPad Prism software.
Statistical significance (*) was determined when $p<0.05$

3.5.2. Interleukin-6 (IL-6)

Median constitutive IL-6 production by primary nasal epithelial cells was 26.2pg/ml, whereas primary type II alveolar epithelial cells produced higher baseline levels: 236pg/ml. Both cell types responded by increasing their secretion of IL-6 in response to TNF-α stimulation, although alveolar cells displayed a statistically significant increase. (Figure 3.28)

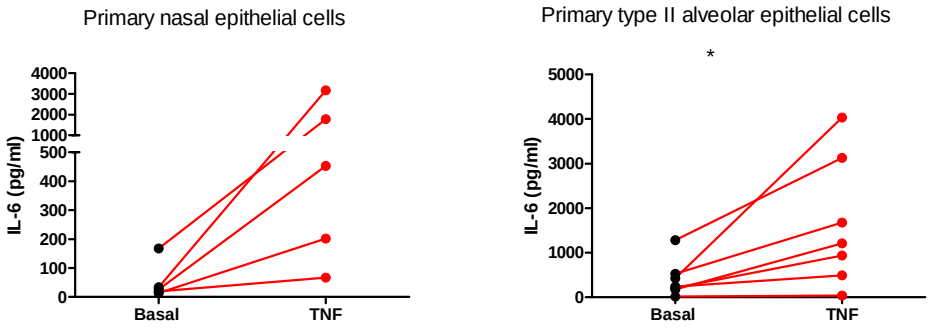


Figure 3.28 IL-6 production by primary nasal epithelial cells and primary type II alveolar epithelial cells.
Cells were treated in a similar way as per Figure 3.43. Cell free supernatants were collected and processed.
Data are presented as individual data points for n=5 for the primary nasal epithelial cells and n=7 for primary type II alveolar epithelial cells.
Statistical analysis was performed using the Wilcoxon matched pairs test using GraphPad Prism software.
Statistical significance (*) was determined when $p<0.05$

3.5.3. Interleukin-10 (IL-10)

Median constitutive levels of IL-10 in supernatant from primary nasal epithelial cells were 10.4pg/ml, while in primary type II alveolar epithelial cells they were 15pg/ml. Stimulation with TNF-α was not associated with an increase in the

production of this cytokine by primary nasal epithelial cells. On the contrary, type II alveolar epithelial cells responded significantly to the TNF-α challenge. (Figure 3.29)

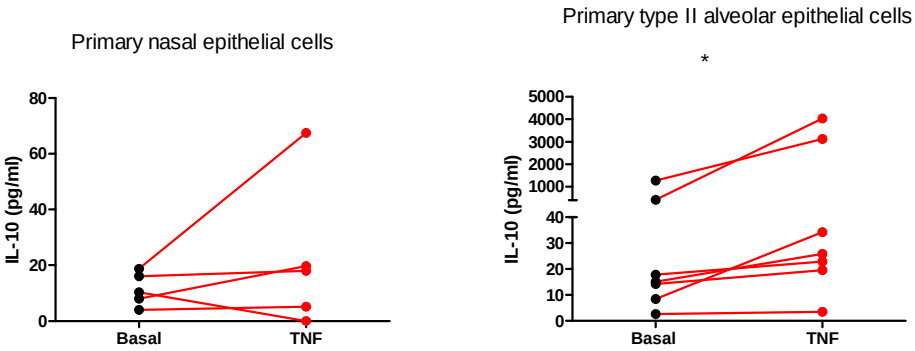


Figure 3.29 IL-10 production by primary nasal epithelial cells and type II alveolar epithelial cells.
Cells were treated in a similar manner as in Figure 3.43.
Data are presented as individual data points for n=5 in nasal primary epithelial cells and n=7 in primary type II alveolar epithelial cells
Statistical analysis was performed using the Wilcoxon matched pairs test using GraphPad Prism software.
Statistical significance (*) was determined when $p<0.05$

Median constitutive levels of IL-1β, TNF-α and IL-12p70 in supernatants of primary nasal and alveolar epithelial cells were low. In both cell types stimulation with TNF-α was not associated with an increase in the secretion of these cytokines.

For a summary of the cytokine responses between cells from the upper and lower respiratory tract, the reader is directed to table 3.7.

Cytokine	Primary nasal epithelial cells Basal (pg/ml) n=5	Primary nasal epithelial cells TNF-α (pg/ml) n=5	P value	Primary type II alveolar epithelial cells Basal (pg/ml) n=7	Primary type II alveolar epithelial cells TNF (pg/ml) n=7	P value
IL-8	282 (192.8-1004)	2975 (2033-48688)	0.0625	2273 (707-11226)	31721 (9450 – 78198)	*0.0156
IL-6	26.2 (13.7-167.8)	452.6 (67.50-3173)	0.0625	236.3 (8.3-1276)	1205 (34.18-4029)	*0.0156
IL-1β	7.1 (0-9.5)	4.2 (0-165)	0.6250	5.2 (2.8-8.1)	11.2 (1.2-85.3)	0.1094
TNF-α	10.3 (1.7-14.9)		N/A	10 (3.6-21.2)		N/A
IL-10	10.4 (4-18.7)	18 (0-67.5)	0.3125	15.2 (2.6-1276)	25.8 (3.5-4029)	*0.0156
IL-12p70	12.6 (3.6-19.8)	6.3 (0-23.3)	1	8 (5.4-19.7)	6.9 (2.7-30.3)	1

Table 3.7 Comparison of cytokine responses between human primary nasal epithelial cells and primary human type II alveolar epithelial cells before and after stimulation with TNF-α
Statistical analysis was performed using the Wilcoxon matched pairs test to compare results for paired samples before and after stimulation using GraphPad Prism software.
Statistical significance (*) was determined when $p<0.05$.

3.6. Discussion

The RPMI 2650 cell line was established by Moore and Sandberg in July 1962 when it was reported as a permanent cell line derived from the pleural effusion of a patient with anaplastic squamous cell carcinoma of the nasal septum. They described its quasi-diploid karyotype, which resembles the normal human epithelium, as well as similarities in the cytokeratin polypeptide profile and the presence of mucoid material on the surface during culture. My findings support their observations except that during culture I did not observe the production of mucus by the cells. My results also support the report made by Moll, R., et al (1983) and De Fraissinette, A., et al, (1995), that RPMI 2650 cells express high levels of CK-18 and CK-7-17, although CK-10 expression was low in my experiments. I also demonstrated that as well as their primary counterparts, both cell types express CK-18 and vimentin, although the expression of the latter appears to be variable in the primary nasal epithelial cells. This finding is also consistent with the study from Kasper, M., and Stosiek, P., in 1990 whom investigated biopsies of normal nasal tissue showing the dual expression of CK-18 as well as vimentin in epithelial cells. The authors argue that the co-expression of cytokeratin and vimentin in epithelial cells is related to the secretory function of non-glandular epithelium in cavities which are in contact with low-protein body fluids.

Electron microscopy studies by Salib, R., et al, 2005, have shown that RPMI 2650 cells are not fully differentiated since they grow in multiple layers and do not possess any cilia. These studies have also been supported by Bai, S., et al, 2008 which showed that one of the limitations of this model is that RPMI 2650 cells are not able to form tight junctions during fluid culture and that they need to be grown at an air-liquid interface in order to develop them. Schmidt, M., et al, 1998 have reviewed *in vitro* models to study nasal mucosal permeability and metabolism and have reported that the limitations of this cell line are the poor differentiation and the lack of polarisation. Studies by Boucher, R., et al, 1987 as well as from Schmidt, M., et al, 1998 have shown that the trans-epithelial electrical resistance in the cell line is similar to that in fresh excised nasal mucosa and is typical of leaky epithelium.

I have demonstrated that the cell line RPMI 2650 actively produces the cytokine TGF-β2 as reported by Carey, B., et al, 1993. This cytokine serves as an autocrine growth factor given not only its active secretion, but also by the presence of TGF-β2 receptors as detected by flow cytometry in the study by Salib, R., et al, 2005. TGF-β is an important pleiotropic cytokine - its functions include the maintenance of tolerance (Schull, M., et al, 1992) through the regulation of T and B lymphocyte responses (Gorelik, L., et al, 2000 and Cazac, B., et al, 2000), as well as having a role in mucosal immunity given by its ability to modulate isotype switching to IgA in mucosal intestinal epithelium (Kim, P.,

and Kagnoff, M., 1990; Ehrhardt, R., et al, 1992). Fong, C., et al, 2000, have, in addition to other groups, shown its role in the release of IL-8 in human airway smooth muscle cells and Pelaia, G., et al, 2002, have shown that TGF-β induces an approximate nine-fold increase in p38 MAPK activation in airway epithelial cells. Of its three isoforms, TGF-β2 appears to be the main isoform expressed in the airways (Balzar, S., et al 2005; Puddicombe,S., et al, 2000) and it has been noted that in these sites this isoform can modulate subepithelial fibroblast function.

The behaviour of the cell line RPMI 2650 is characterised by its inability to constitutively produce the cytokines IL-6, IL-8 and the antimicrobial peptide SLPI. IL-10 production was observed at low levels. The fact that these cells appeared refractive to stimulation with bacterial TLR ligands such as LPS, PGN and LTA, as well as to the pro-inflammatory cytokine TNF-α suggests that they could have lost TLRs as well as TNF-α receptors as a result of the multiple passages that they had gone through, although upon receipt of the vial, multiple frozen aliquots were prepared and the cells were discarded after passage number 20. My findings agree with those of Lindbom, J., et al, 2006 when studying the cytokine release by human macrophages and respiratory cells after exposure to particles generated from studded tires and pavement using the nasal epithelial cell lines (RPMI 2650) and bronchial epithelial cells (BEAS-2B). They reported that none of the particle types used in their experiments were able to evoke a

detectable cytokine release from RPMI 2650 cells. They attributed their findings to the transformation process of these cells into immortalized cell lines. Another option could be that since RPMI 2650 cells constitutively produce high levels of TGF-β2, that the over-expression of receptors for this cytokine could inhibit the cellular activation given by TLR ligands, although this is unlikely since, as discussed above, this cytokine favours a pro-inflammatory phenotype through the increase in p38 MAPK activation.

In contrast, primary nasal epithelial cells were able to produce constitutive levels of cytokines such as IL-8 and IL-6, and stimulation with the bacterial ligand PGN from *S. aureus* invariably induced an increase in the release of these cytokines, as did stimulation with the pro-inflammatory cytokine TNF-α. These findings are in accordance with those reported by McDougall, C., et al, 2008, which showed a significant increase in the release of these cytokines after stimulation with 10ng/ml of TNF-α by primary nasal epithelial cells. Constitutive production of the cytokines IL-1β, TNF-α, IL-10 and IL-12p70 was very low, just above the detection limit for the assay and I did not observe an increase in the secretion of these cytokines after stimulation with TLR ligands or with TNF-α. It is interesting to note that stimulation of these cells with LPS was not associated with an increase in the secretion of IL-8 and IL-6. A study by Wang, J., et al, 2007, observed the involvement of TLR in nasal polyp epithelial cells from patients with chronic rhinosinusitis. They measured IL-8, RANTES, IP-10

and GM-CSF, and found that these cells did not respond significantly to stimulation with 10μg/ml of PGN, whereas they responded significantly to stimulation with 10μg/ml of *E. coli* LPS, although they also reported that their predominant finding was the high response to double stranded RNA (dsRNA), an activator of TLR3. Chronic rhinosinusitis is usually precipitated by viral infections and that could explain their results since these patients' cells could be primed by repeated viral infections. In contrast, in my work the primary nasal cells were obtained from patients with no previous history of nasal pathology and therefore reflect the response of a relatively healthy mucosa, although all of the patients had previous exposure to smoking. The increase in the cytokine response after stimulation of the cells with PGN from *S. aureus* (which is a well recognised bacterium present in the anterior nasal cavity (Gluck, U., and Jebbers, J., 2000)) suggests that the nasal epithelium provides a surveillance mechanism for the presence of pathogens. This is supported by the study by Ritz, H., et al, 1984 in which healthy volunteers colonised with toxic shock antigen(TSA) producing strains of *S. aureus* were found to have high levels of serum antibodies to TSA. The lack of response of the cells in my system to LPS in comparison to the Wang study could be related to the lower dose of LPS used (100ng/ml) as well as to the different concentrations of serum in the different culture systems, although they also reported in their study a low expression and functionality of TLR2 and TLR4. To my knowledge this is the first report trying

to characterise the cytokine response to bacterial ligands in primary nasal epithelial cells.

Co-culture experiments with *S. aureus* failed to provide useful information regarding how this bacterium establishes colonisation of the nasal epithelium due to its aggressive behaviour. They also demonstrated the need for developing a sterile model in order to try to mimic the *in-vivo* environment in which the effector cells of the innate immune system provide support to the epithelial cells in order to maintain a low bacterial number which prevents overwhelming bacterial growth. Using QPCR it was possible to observe the multiple effects that *S. aureus* infection induces in cellular metabolism.

The dynamics of *S. aureus* infection in airway epithelial cells have been studied by da Silva, M., et al, 2004. Their study shows that after a 24 hour infection period with *S. aureus*, almost 90% of the airway cells exhibited severe damage of the cellular membrane. Further experiments demonstrated that the cells underwent apoptosis first rapidly followed by necrosis. In their study airway epithelial cells exhibited efficient defence mechanisms against *S. aureus* as evidenced by the permeabilisation of the plasma membranes in almost 50% of the bacteria detected in cell culture supernatants 1hour post-infection. However supernatants retrieved from the cells at 24 hours exhibited a lower capacity to inhibit growth of *S. aureus*, suggesting that during infection the self-defence

mechanisms of airway cells may have been overwhelmed. My experiments are similar to those of da Silva et al in that the cells were kept continuously exposed to *S. aureus* in suspension in the culture medium and under these conditions extracellular bacteria are likely to have replicated and released virulence factors. Because I did not wash the cells after 1 hour of infection the effects happened in a more speedy manner in my system. Furthermore, the data presented by Bantel, H., et al, 2001 showed that in the induction of cell death by *S. aureus* α-toxin, necrosis predominated despite the apoptotic activation of caspase 8. Kahl, B., et al, 2000, have shown that internalised *S. aureus* replicates actively inside pulmonary epithelial cells inducing apoptosis. Intracellular replication induces apoptosis. There is growing evidence that *S. aureus* may be internalised into epithelial cells (Bayles, K., et al, 1998; Kahl, B., et al, 2000). The advantages of the intracellular location for the bacteria are the ability to avoid host defence mechanisms as well as antibiotics. In the study by da Silva and colleagues, they found that *S. aureus* was efficiently internalised by airway epithelial cells in a time and dose dependent fashion. They found that *S. aureus* replicates actively inside pulmonary epithelial cells and they also showed that infected airway epithelial cells showed evidence of apoptosis with typical DNA laddering, alteration of cellular morphology and annexin V staining occurring at 24h but not at 4h after infection. The co-localisation of green fluorescent bacteria inside apoptotic cells in these experiments suggests that bacterial replication precedes and induces apoptosis. Apoptosis was not induced in cells where internalisation

was blocked by cytochalasin D or in cells infected with heat killed bacteria. Taken together, these observations illustrate the devastating and multiple effects that live infection cause in cellular physiology and support the concept of moving onto a sterile model in which processes could be studied in a more controlled fashion.

In comparison to the nasal epithelial cell line RPMI 2650, the cell line A549 was found to produce constitutively lower levels of TGF-β2, and constitutive production of IL-8 was higher than IL-6 as reported by Burvall, K., et al., 2003 After stimulation with PGN and TNF-α, IL-8 and IL-6 secretion increased significantly. Median IL-10 production by this cell line was low but increased after TNF-α stimulation in a non-significant manner. These results are in accordance with Burvall et al who demonstrated that A549 cells produce constitutively low levels of IL-6 and higher levels of IL-8. In comparison to the RPMI 2650 cell line, the A549 cell line appears more responsive to TLR and TNF-α ligand stimulation, nevertheless, it is interesting to note that this cell line failed to respond to stimulation with LPS. This observation has also been reported by Guillot, L., et al, 2004 who demonstrated the intracellular compartmentalisation of TLR4 using the same cell line, as well as the human tracheobronchial epithelial cell lines BEAS-2B and 16HBE. Using flow cytometry techniques they demonstrated that TLR4 is not expressed at the surface of the cell lines A549 and BEAS-2B. They also provided evidence for

intracellular TLR4 localisation in human primary bronchial epithelial cells and that the production of the cytokines IL-8 and IL-6 acts is dose-dependent, with the highest secretion being found when they were stimulated with 10μg/ml of LPS. Previous evidence from Guha, M., and Mackman, N., 2001; Zarember, K., et al, 2002, has shown that phagocytic cells are highly sensitive to the LPS dose as they respond with increase secretion of proinflammatory cytokines when they are stimulated by 1-10ng/ml of LPS. My results are in agreement with this finding since stimulation of the cell line A549 at 10ng/ml and 100ng/ml was not associated in an increase in the secretion of cytokines in supernatant. The study by Bérubé, J., et al, 2009 also used LPS at 100ng/ml to stimulate BEAS-2B cells, a human cell line derived from normal bronchial epithelium. Their results showed only a modest increase in IL-8 secretion in supernatants after 24 hours of stimulation, the predominant responses being after stimulation with Pam3CSK4 (an agonist of TLR1/2) poly I:C (a TLR3 agonist) and C12-ie-DAP (an acylated derivative from peptidoglycan which binds the intracellular NOD1 receptor). Similar results were obtained when I stimulated human primary type II alveolar epithelial cells. Taken together these findings suggest that initiation and signalling events appear to be less efficient in pulmonary cells. Hornef, M., et al, 2002, have demonstrated the intracellular distribution of TLR4 in the Golgi apparatus, where it co-localises with internalised LPS, and its absence on the surface of intestinal epithelial cells, in contrast to its membrane expression in monocytes. This intracellular compartmentalisation could be a negative

regulation mechanism in order to avoid excessive activation of pulmonary epithelial cells due to their regular exposure to low amounts of LPS in the air.

On the other hand, one of the main consistent findings of this research was the significant increase of cytokine production by the A549 cell line and by primary human nasal and type II alveolar epithelial cells after stimulation with *S. aureus* PGN. Hussain, T., et al, 2008 have shown that PGN up-regulates TLR-2 expression in mouse primary pleural mesothelial cells. Using the alveolar epithelial cell line A549 Cheon, I., and coworkers, (2008) demonstrated that *S. aureus* PGN induces IL-8 mRNA and protein expression in a dose and time dependent manner. Furthermore, they also demonstrated that PGN increases the DNA binding of the transcription factors AP-1 and NF-κB. Leemans, J., et al, 2002, studied the differential role of LTA and PGN from *S. aureus* in the induction of lung inflammation in bronchoalveolar lavage (BAL) from IL-$6^{+/+}$ BALB/c mice and IL-$6^{-/-}$ mice on a BALB/C background; their data suggest that intrapulmonary administration of LTA provokes an inflammatory response that is very similar to that induced by LPS showing an increase in PMN and elevated BAL concentrations of TNF-α and IL-6. While inoculation of PGN resulted in increase PMN infiltration and cytokine concentration, histologically it induced the formation of numerous abscesses centred around small bronchi and mostly composed of PMN, quite different from the LTA-induced interstitial inflammatory infiltrate in the lungs. Hessle, C., et al, 2005 have shown that PGN

enhances the production of pro-inflammatory cytokines to a greater degree than LTA in human monocytes. The specific signalling pathways are still controversial. The study by Schwandner, R., et al, 1999, suggested that PGN is recognised by TLR2, while Travassos, L., and coworkers, (2004) have argued that highly purified PGN is not sensed by TL2, TLR1/2 or TLR2/6. Furthermore, Strober, W., et al, 2006, have shown that Gram-positive bacterial PGN preferentially activates NOD2. Girardin, S., et al, 2003, have shown that PGN is identified by NOD2 through the recognition of muramyl dipeptide (MDP).. Further work should establish the presence of this receptor in primary type II alveolar epithelial cells and aim to dissect the pathways associated with the different responses.

Despite several unsuccessful attempts it was possible to isolate and culture primary human lung fibroblasts and primary type II alveolar epithelial cells from 8 patients that underwent lung resection. The phenotype of the primary type II alveolar epithelial cells was confirmed biochemically by positive staining for alkaline phosphatase which confirmed the purity of the monolayers used for further experimentation, and by molecular methods identifying transcripts for surfactant protein-C and aquaporin-3.

It was possible to detect the cytokines IL-8, IL-6, IL-1β, TNF-α, IL-10 and IL-12p70 in supernatants from unstimulated human primary type II alveolar

epithelial cells. Upon stimulation with bacterial ligands, particularly with *S. aureus* PGN, there were significant increases in the production of the pro-inflammatory cytokines IL-8, IL-6, Il-1β, and TNF-α. It is interesting to note that in my system the primary type II alveolar epithelial cells did not respond to stimulation by the TLR-2 agonist LTA. This result agrees with the report published by Mayer, A., et al, 2007, in which they noted that stimulation of human bronchial epithelial cells with 10μg/ml of LTA was not associated with an increase in IL-8 production, probably related to the low expression of TLR2 by bronchial epithelial cells when compared to monocytes. Upon transfection with TLR2 responsiveness to LTA was observed. Their results support the concept that in airway epithelium low TLR2 expression might play a role in limiting uncontrolled activation by inhaled bacteria.

Human primary type II alveolar epithelial cells also responded with a significant increase of the pro-inflammatory cytokine TNF-α as expected. It was also observed that there were no changes in the production of the cytokine IL-12p70 and this might reflect the purity of the epithelial cell preparations, since this cytokine is mainly produced by myeloid cells particularly human monocytes as well as peripheral blood myeloid dendritic cells (Bekeredjian-Ding, I., et al, 2006). Another observation worthy of mention is the lack of response of primary type II alveolar epithelial cells to stimulation with an ultrapure preparation of *P. aeruginosa* LPS. As discussed above, this could be due to the intracellular

localisation of the TLR4 in these cells. The study by Armstrong, L., et al, 2004, has demonstrated functional responses to LPS and LTA, although these depend on higher concentrations of these ligands and are of a smaller magnitude than the responses in monocytes and macrophages, The differences between my results and theirs are probably related to the LPS dose used in their study (10μg/ml whereas in my study it was 100ng/ml). The LPS used in my study was also an ultrapure preparation of LPS which is highly unlikely to contain contaminant lipoproteins. It is also relevant to mention that the cells were obtained from previous smokers who underwent resection for lung cancer. MacRedmond,R., et al, 2007 have shown a dose-dependent down-regulation of TLR4 mRNA and protein expression as a result of stimulation with smoke extracts in A549 cells, and it is highly likely that a similar thing happens in my system. On the contrary, the study by Togbe, D., et al (2006) using TLR4 transgenic mice showed that over-expression of TLR4 augmented a LPS-induced bronchochostrictive effect as well as TNF-α production. They also found increased PMN recruitment and microvascular and alveolar epithelial injury depending on the *TLR4* gene dose. These findings suggest that the down-regulation of TLR4 in the airway epithelium is a key regulatory physiological mechanism in order to avoid an excessive inflammatory response in the lungs.

When comparing the A549 and RPMI 2650 cell lines it was observed that RPMI 2650 cells' main feature was the inability to constitutively produce cytokines and

to respond to stimulation and this could be due to loss of receptors. I also observed lack of mucus production as compared with the original report from Moorehead, P., 1965. The A549 cell line appears still to retain some of the original features of primary type II alveolar epithelial cells. On the other hand their inability to express surfactant protein-C and AQP3 suggest that multiple passages have altered the original phenotype. This is supported by the work of Tetley's group in which they did not find transcripts for these genes (Witherden, I., et al, 2004). Taken together, these findings support the idea that making assumptions of normal physiological behaviour is difficult using these cell lines as models.

When comparing the response of the primary nasal epithelium to the alveolar epithelium it was observed that the lung epithelium seems to display a pro-inflammatory phenotype relative to the nose epithelium, since basal IL-8 and IL-6 levels were significantly higher in alveolar cells. Stimulation with *S. aureus* ligands such as peptidoglycan and lipoteichoic acid was associated with significantly higher levels of IL-8 and IL-6 in the alveolar cells, and there was a discrete increase also in the levels of IL-1β production, although these were not statistically significant. Other cytokines such as TNF-α, IL-10 and IL-12p70 showed no differences in their profiles when the primary nasal and alveolar cells were compared. A lack of response after stimulation with *P. aeruginosa* LPS was also observed suggesting that the refractivity of these cells appears to be related

to their respiratory origin and that this response is probably the result of evolution, in view of the ubiquitous nature of LPS in the environment. Primary nasal epithelial cells constitutively produce low levels of pro-inflammatory cytokines and these cells are responsive to stimulation. Their baseline production levels are lower in comparison to type II alveolar epithelial cells. These latter cells not only behave in a more pro-inflammatory pattern but appear mucho more responsive to PGN by inducing a significant rise in concentrations of IL−1β, IL-6, IL-8, IL-10 and TNF. These findings support the hypothesis that the alveolar epithelium behaves in a more pro-inflammatory way and that these cells are likely to exert not only a higher surveillance mechanism, but also to act as key orchestrators of a pro-inflammatory environment as observed during clinical pneumonia. The nasal epithelium, although able to elicit inflammatory responses after stimulation, appears less responsive and therefore is likely to be more tolerant to the presence of bacteria.

Taken together, the findings in primary cells support clinical observations. Further work is needed to elucidate the precise molecular mechanism behind these responses in order to assess constitutive differences in the presence of TLRs in both cell types and whether primary nasal epithelial cells express repressor proteins or have different requirements for activation of the transcription factor NF-κB.

CHAPTER FOUR

RESULTS

Results

4.1 Toll-like receptors in the respiratory tract

4.1.1 Introduction

The airways are the first port of entry of microorganisms and foreign matter into the respiratory system. Increasing evidence is placing the airway epithelium as a key orchestrator of innate immune responses to infection (Xiang, M., and Fan, J., 2010). Toll-like receptors constitute one of the main sources of surveillance in cells of the innate immune system. Upon engagement of the receptors a series of signals are followed in order to activate the immune system in response to the presence of invading microorganisms. These receptors have been extensively characterised in leukocytes (Aderem, A., and Ulevitch, R., 2000 and Mogensen, T., 2009) as well as in other organ systems like the gastrointestinal tract (Otte, J., et al, 2004 and Melmed, G., et al, 2003). Their presence and function in the respiratory tract has been an area of increasing interest, and they have been studied and characterised in cells of the lower respiratory tract (Gentry, M., et al 2007, Armstrong, L., et al, 2004, Droemann, D., et al, 2003). However, nasal cells have received less attention. My objective was to describe the characteristics of the main TLRs involved in the response to bacterial infection in human primary nasal epithelial cells.

TLR signalling is at the core of innate immune responses and its regulation deserves a great deal of attention since multiple disease processes have been associated with excessive TLR-driven responses, such as sepsis (Agnese, D., et al,

2002, Lorenz, E., et al, 2002) and autoimmunity (Tao, K., et al, 2007). I also wanted to explore and characterise the presence of toll-interacting protein (tollip) in the respiratory tract. Tollip is a protein involved in the negative regulation of TLR-associated responses which has been found to be over-expressed in cells of the gastrointestinal tract which are unresponsive to the vast amount of bacteria that lie within (Otte, J., et al, 2004 and Melmed, G., et al, 2003).

The hypothesis that I would like to test are:

1. ***Tollip is expressed in the human respiratory tract***
2. ***Tollip expression differs between the upper and the lower respiratory tract***

4.2 TLRs in primary human nasal epithelial cells

The presence of TLRs in human nasal epithelial cells has largely been determined looking at nasal polyp tissue (Wang, J., et al, 2007, VanderMeer, J., et al, 2004 and Lane, A., et al, 2006). Although these studies have made an important contribution to the understanding of innate immunity in the nasal cavity, nevertheless the tissue used in their experiments is far from normal and therefore I wanted to study the presence of these receptors in nasal brushings from healthy volunteers without a previous history of nasal pathology.

4.2.1 Toll-like receptor 1 (TLR1)

The presence of Toll-like receptor 1 in primary human nasal epithelial cells was determined using confocal microscopy. Previous reports in the literature by Wyllie, D., et al, 2000 and Hornung, V., et al, 2002, have provided evidence of the presence of this receptor on the cell surface of primary human monocytes. These cells from healthy donors were therefore used as a positive control and subsequently I assessed the presence of TLR1 in primary human nasal epithelial cells. In monocytes, the presence of TLR1 was evidenced by membrane staining. When primary human nasal epithelial cells were examined, there was a diffuse cytoplasmic stain, suggesting that primary nasal epithelial cells express TLR1 more abundantly than expected (Figure 4.1).

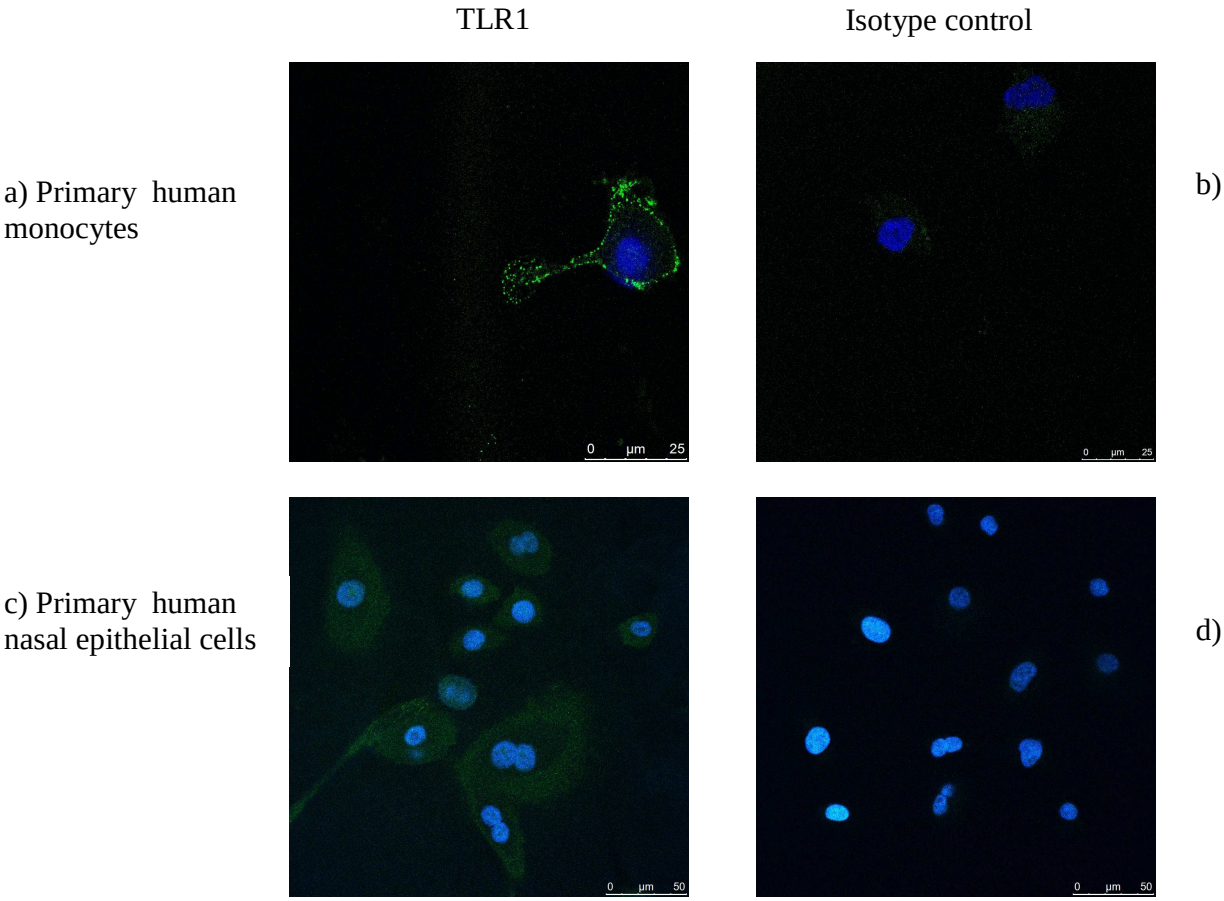


Figure 4.1 Detection of Toll-like receptor 1 (TLR1) in human primary monocytes and primary nasal epithelial cells.
Cells were seeded onto coverslips for 24 hours, then fixed with methanol, blocked with 2% goat serum and incubated with a monoclonal mouse antibody against TLR1. Nuclei were stained with DAPI (blue). Secondary antibody was anti-mouse IgG conjugated with Alexa 488 (green).
Images were analysed using confocal microscopy. a) primary blood monocytes and TLR1, b) primary blood monocytes and isotype control, c)primary human nasal epithelial cells and TLR1, d) primary human nasal epithelial cells and mouse isotype control.
Scale bar equals 25µm in monocyte images to illustrate the detail of the membrane staining with TLR-1, 50µm in primary nasal epithelial cells to illustrate the diffuse abundance of the cytoplasmic stain.

4.2.2 Toll-like receptor 2 (TLR2)

The presence of TLR2 has been described in monocytes by Hornung, V., et al (2002) and Parker, L., et al, 2004. I assessed the presence of this receptor in primary blood monocytes using confocal microscopy. An abundant cytoplasmic stain was observed, when compared to primary human nasal epithelial cells. The stain was also cytoplasmic in nasal cells but much less strong in comparison to that in primary monocytes. (Figure 4.2)

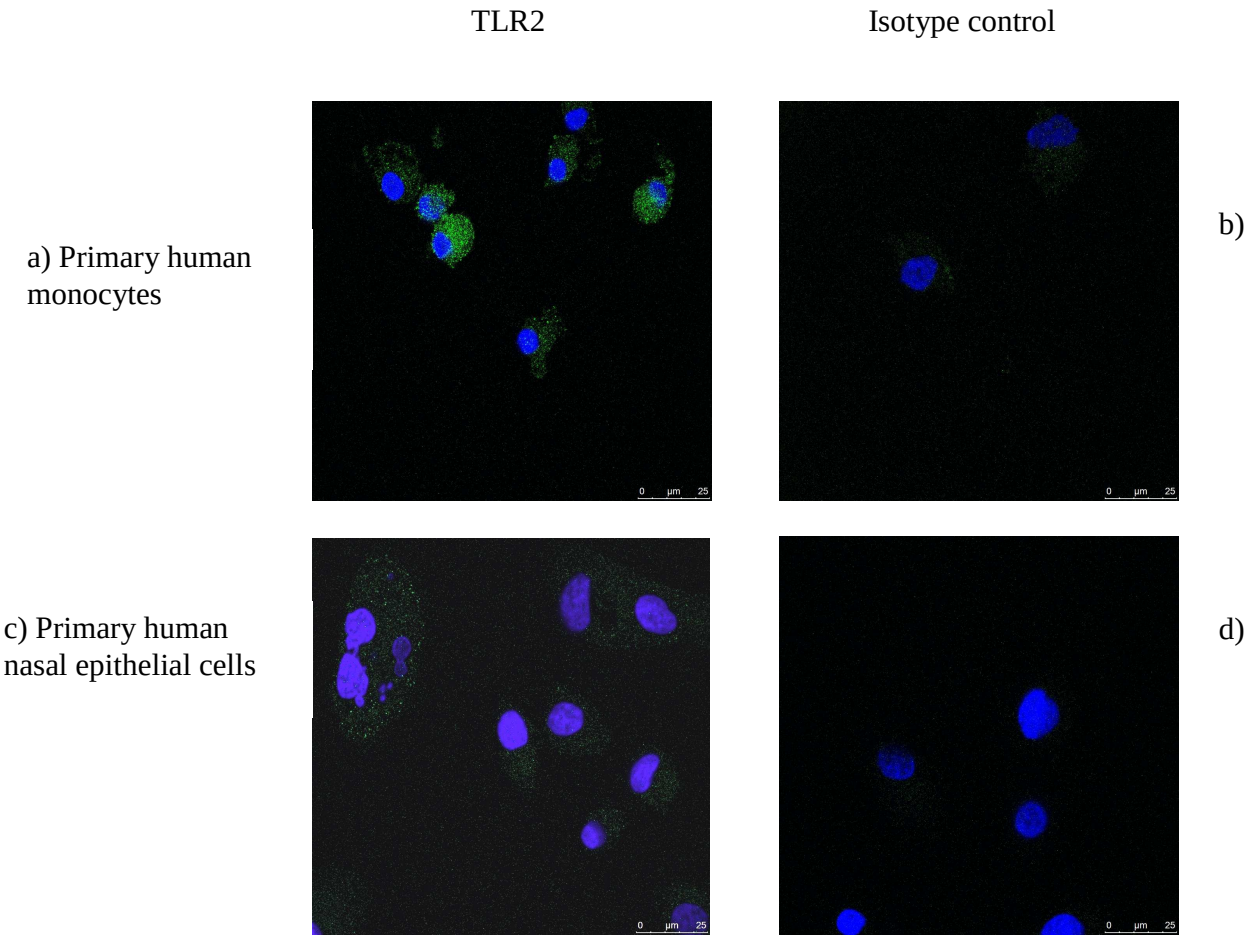


Figure 4.2 Detection of Toll-like receptor 2 (TLR2) in human primary monocytes and primary nasal epithelial cells.
Cells were seeded onto coverslips for 24 hours. They were then fixed with methanol, blocked with 2% goat serum and incubated with a monoclonal mouse antibody against TLR2. Nuclei were stained with DAPI (blue). Secondary antibody was anti-mouse IgG conjugated with Alexa 488 (green).
Images were analysed using confocal microscopy: a) primary human monocytes and TLR2, b) primary human monocytes and mouse isotype control, c) primary human nasal epithelial cells and TLR2, d) primary human nasal epithelial cells and mouse isotype control.
Scale bar equals 25μm.

4.2.3 Toll-like receptor 4 (TLR4)

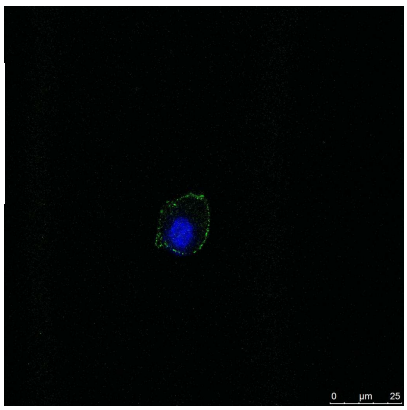
TLR4 was the first receptor of the TLR family to be discovered by Charles Janeway Jr. in 1997. Since then multiple groups have demonstrated the distribution of this receptor in multiple cell types and tissues. The presence of this receptor in primary human monocytes has been described by Lien, E., and Ingalls, R., (2001), Hornung, V., et al, 2002, and Parker, L., et al, 2004, among others.

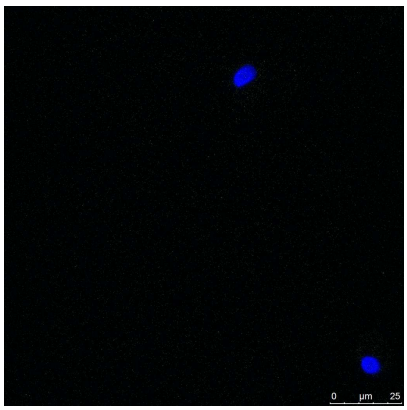
TLR4 was identified in primary nasal epithelial cells using confocal microscopy. It was possible to observe different types of staining within the same sample of cells. This suggests that some cells express this protein more strongly than others Staining was predominantly intracellular, as opposed to membrane-associated. (Figure 4.3)

TLR4

Rabbit IgG Control

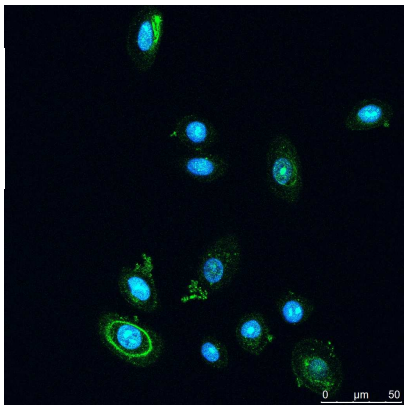
a) Primary human monocytes

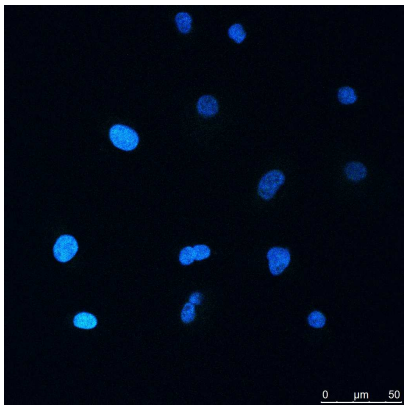




b)

c) Primary nasal epithelial cells





d)

e) Primary nasal epithelial cells

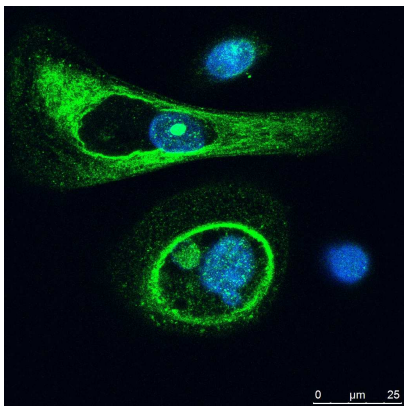


Figure 4.3 Detection of Toll-like receptor 4 (TLR4) in human primary monocytes and nasal epithelial cells.
Cells were treated in a similar way as described for the previous receptors. After fixation and blocking, the cells were incubated with a polyclonal rabbit antibody against TLR4. Nuclei were stained with DAPI (blue). Secondary antibody was anti-rabbit IgG conjugated with Alexa 488 (green).
Images were analysed using confocal microscopy. a) primary human monocytes and TLR4; b)primary human monocytes and rabbit IgG; c)nasal epithelial cells and TLR4 stain, d) primary human nasal epithelial cells and TLR4 higher magnification, e) primary human nasal epithelial cells and control: rabbit IgG.
Scale bar equals 25μm in images a,b and e and 50μm in image c and d.

4.2.4 Toll-like receptor 6 (TLR6)

Toll-like receptor 6 was identified by Akira's group in 1999 and m-RNA transcripts were detected in monocytes, monocyte-derived dendritic cells and neutrophils by Hornung, V., et al (2002) and Hayashi, F., et al (2003). In 2005 Nakao, Y., and colleagues, produced evidence of its expression on the cell surface of primary human monocytes.

In my experiments it was not possible to identify the presence of TLR6 in primary human monocytes, nor was it identified on primary nasal epithelial cells (Figure 4.4).

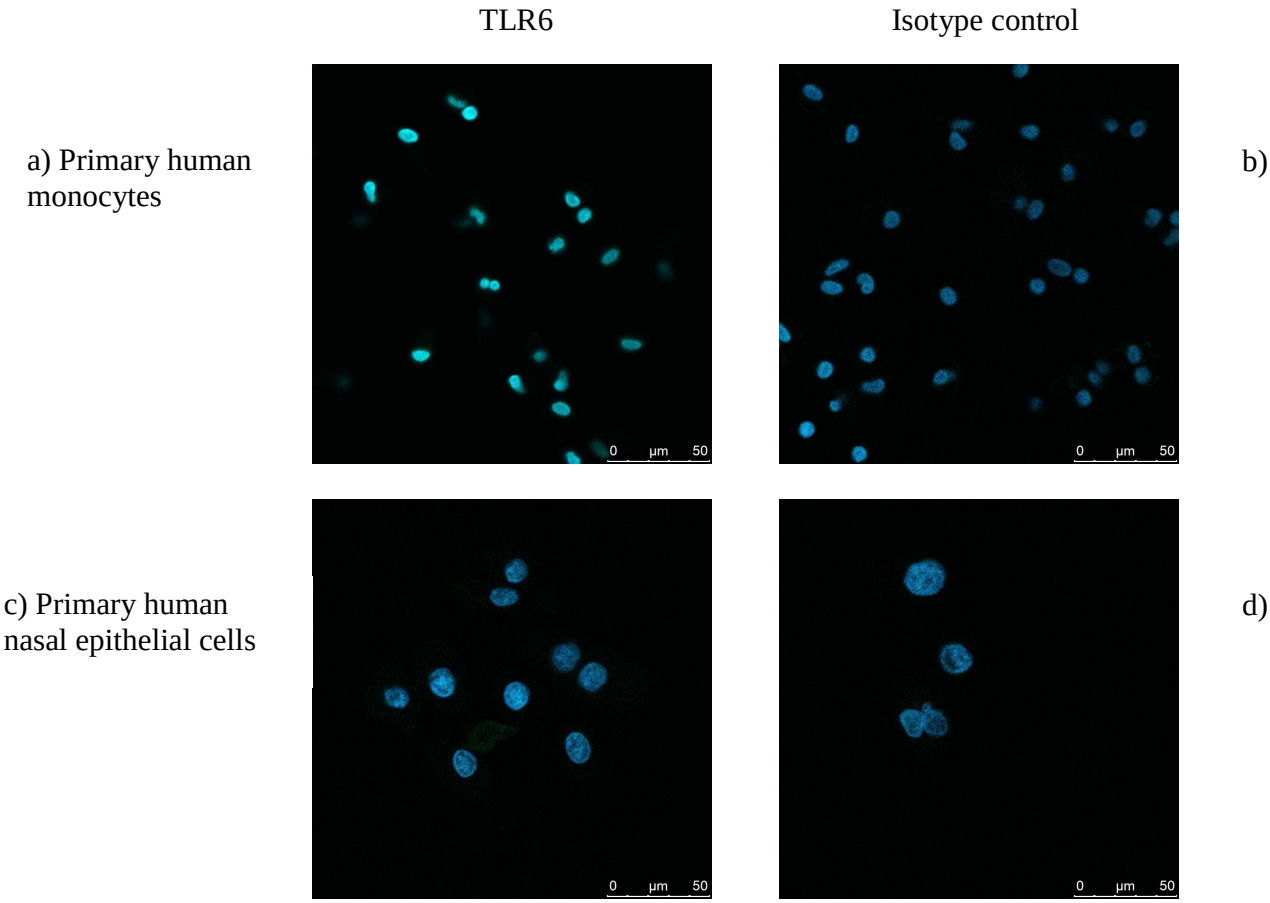


Figure 4.4 Detection of Toll-like receptor 6 (TLR6) in human primary monocytes and human primary nasal epithelial cells.
Cells were treated in a similar way as described for the previous receptors. After fixation and blocking, cells were incubated with a mouse monoclonal antibody against TLR6. Nuclei were stained with DAPI (blue). Secondary antibody was anti-mouse IgG conjugated with Alexa 488 (green).
Images were analysed using confocal microscopy: a)primary monocytes and TLR6 stain, b) primary monocytes and mouse isotype control, c) primary human nasal epithelial cells and TLR6, d) primary human nasal epithelial cells and mouse isotype control.
Scale bar equals 50µm.

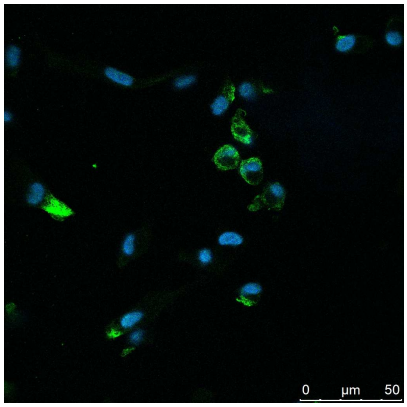
4.2.5 Toll-like receptor 9 (TLR9)

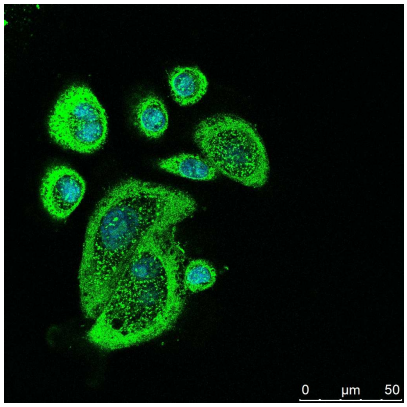
The presence of TLR9 in primary human peripheral blood mononuclear cells was demonstrated by Hornung, V., et al, 2002, using quantitative real-time PCR. In my study, the identification of TLR9 in primary human monocytes was observed as a strong membrane and cytoplasmic stain of different degrees of intensity within the same population of cells, suggesting that expression of this receptor varies among monocytes. When primary nasal epithelial cells were assessed, the stain was abundantly present within all cells in the sample. The stain was predominantly cytoplasmic, and it was interesting to observe a granular pattern distributed within the cytoplasm (Figure 4.5).

Primary human monocytes

Primary human nasal epithelial cells

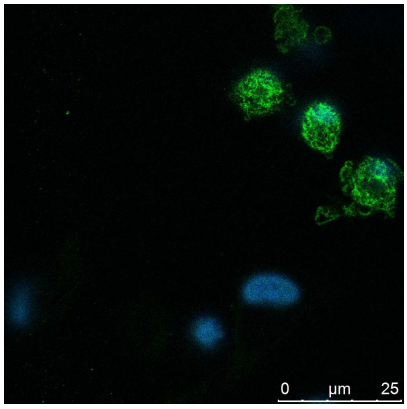
a) TLR9

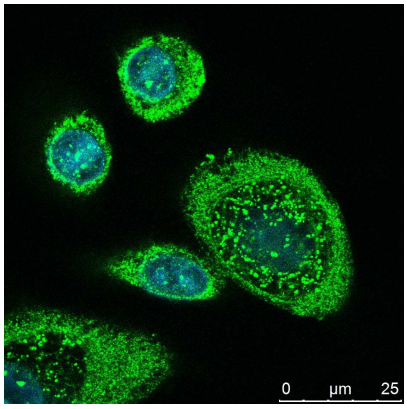




d)

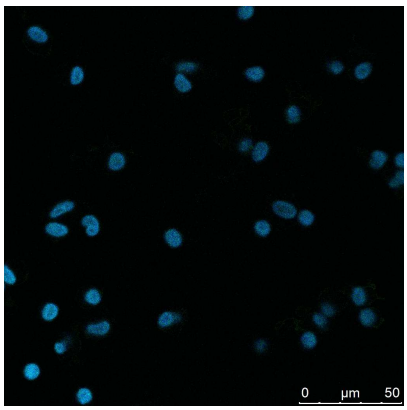
b) TLR9

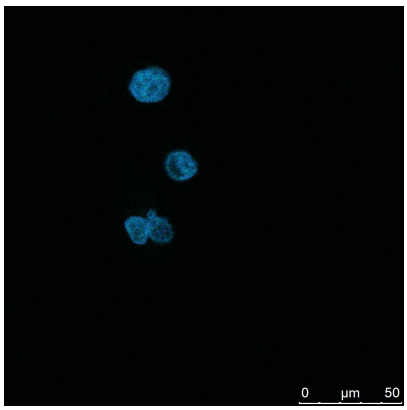




e)

c) Mouse isotype control





f)

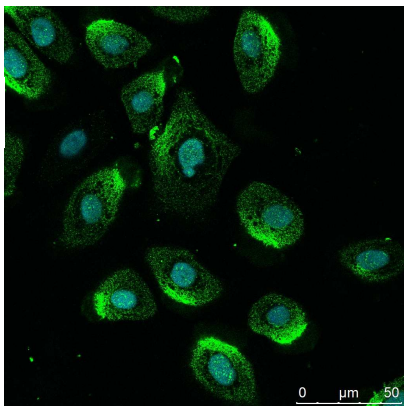
Figure 4.5 Detection of Toll-like receptor 9 (TLR9) in primary human monocytes and primary human nasal epithelial cells.
Cells were treated in a similar manner as described for the previous receptors. After fixation and blocking, cells were incubated with a monoclonal mouse antibody against TLR9. Nuclei were stained with DAPI (blue). Secondary antibody was anti-mouse IgG conjugated with Alexa 488 (green).
Images were analysed using confocal microscopy: a) primary human monocytes and TLR9, b)primary human monocytes and TLR9 (higher magnification), c)primary human monocytes and mouse isotype control, d) primary human nasal epithelial cells and TLR9, e) primary human nasal epithelial cells and TRL9 (higher magnification), f) primary human nasal epithelial cells and mouse isotype control.
Scale bar equals 50μm and 25μm in detailed microphotographs.

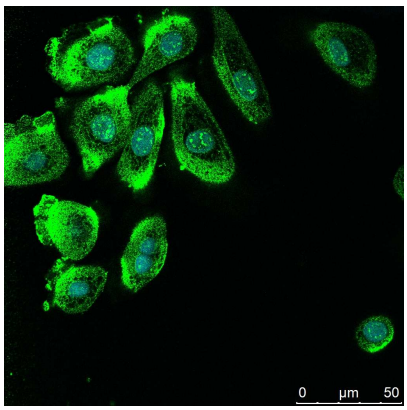
4.2.5.1 Role of TLR9 in primary nasal epithelial cells

4.2.5.1.1 Immunocytochemistry

Since the expression of toll-like receptor 9 (TLR9) was so abundant in primary nasal epithelial cells, I decided to ask if this expression was affected by stimulation of the cells with bacterial ligands such as LPS, LTA, PGN and CpG (bacterial DNA), or with the pro-inflammatory cytokine TNF-α. There were no significant differences of the cells after stimulation, as assessed by confocal microscopy analysis (Figure 4.6).

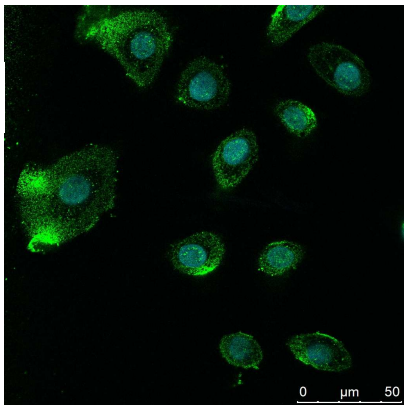
a) Untreated

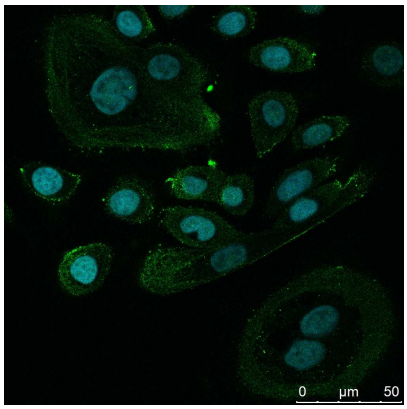




b) LPS

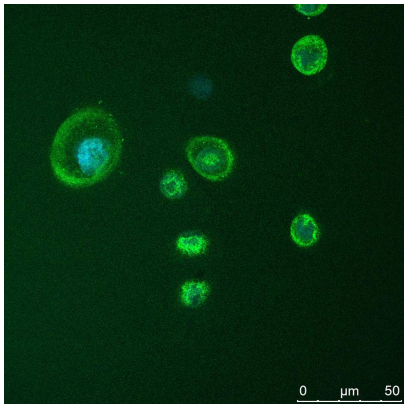
c) PGN

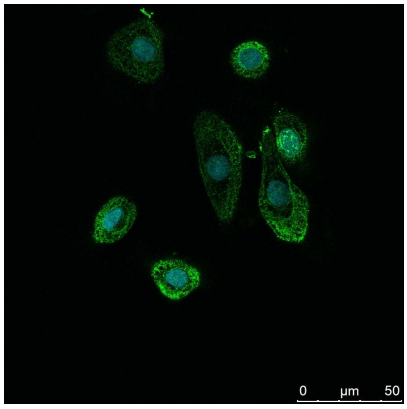




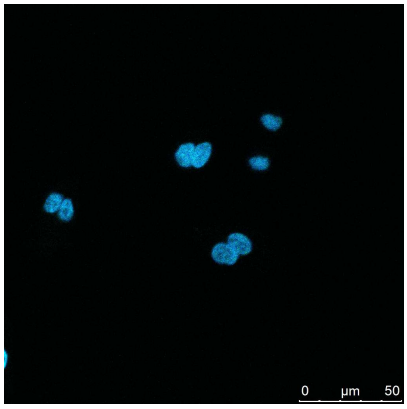
d) LTA

e) CpG





f) TNF



g) Isotype control

Figure 4.6 Regulation of Toll-like receptor 9 (TLR9) in human primary nasal epithelial cells after stimulation with TLR ligands.
Cells were seeded onto coverslips for 24 hours and were left untreated or stimulated for a further 24 hours with bacterial ligands. They were then fixed with methanol, blocked with 2% goat serum and incubated with a mouse monoclonal antibody against TLR9. Nuclei were stained with DAPI (blue). Secondary antibody anti-mouse IgG conjugated with Alexa 488 (green).
Images were analysed using confocal microscopy. a)untreated, b)*P. aeruginosa* LPS, c) *S. aureus* PGN, d) *S. aureus* LTA, e)CpG, f)TNF and g)mouse isotype control.
Scale bar equals 50µm.

4.2.5.1.2 Cytokine responses after TLR9 stimulation of primary nasal epithelial cells

After the demonstration of strong expression of TLR9 in primary human nasal epithelial cells, cells from 5 patients undergoing lung resection were cultured and stimulated with the oligodeoxynucleotide (ODN) 2395 CpG-C DNA, a known agonist of TLR9 (Vollmer, J., et al, 2004 and Sivory, S., et al, 2006). After stimulation, cell-free supernatants were recovered and stored at -80^{o}C until assayed using a CBA kit. The cytokines measured include: IL-8, IL-6, IL-1β, IL-10, TNF-α and IL-12p70. Patient demographics are included in table 4.1

Subject	Age	Sex	Smoker
Patient 3	66	M	Y
Patient 6	57	M	Y
Patient 9	64	F	Y
Patient 20	57	M	Y
Patient 23	69	F	Y

Table 4.1 Characteristics of the five patients from which primary nasal epithelial cells were isolated

The cytokine response by primary human nasal epithelial cells after stimulation of TLR9 with CpG-C DNA produced variable results: while 2 subjects appeared to respond by increasing the secretion of IL-8 and IL-6 in supernatants, the remaining three subjects behaved in a different manner by down-regulating the secretion of these cytokines. The responses to the other cytokines produced highly variable results.

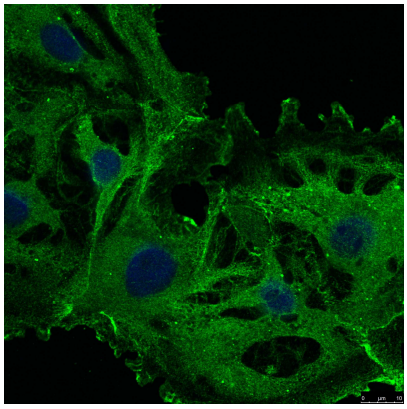
4.2.5.2 Role of TLR-9 in primary human type II alveolar epithelial cells

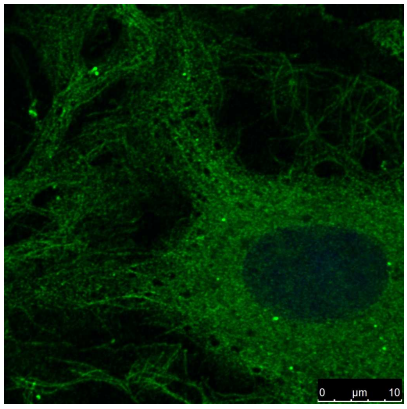
4.2.5.2.1 Immunocytochemistry

Primary human type II alveolar epithelial cells were prepared as described on page 62 in the Materials and Methods section 2.2.3. As these cells require a bovine type I collagen matrix to be cultured, they were seeded onto pre-coated

coverslips. It is possible to observe the strong cytoplasmic uptake of the TLR9 stain (Figure 4.7).

a)





b)

c)

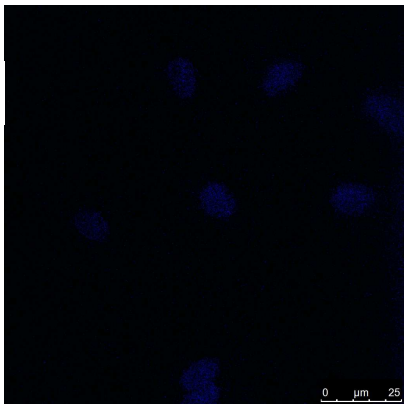


Figure 4.7 Detection of Toll-Like Receptor 9 in primary human type II alveolar epithelial cells.
Cells were isolated as described in the Materials and Methods section and seeded onto glass coverslips pre-coated with type I bovine collagen. Cells were then fixed with 4% paraformaldehyde, permeabilised with 0.1% Triton X, washed, blocked with 2% goat serum and incubated with a mouse monoclonal antibody against TLR-9. Nuclei were stained with DAPI (blue). Secondary antibody was anti-mouse IgG conjugated with Alexa 488 (green).
Images were analysed using confocal microscopy: a) TLR9, b)TLR9 and c)Isotype control.
Scale bar equals 10µm in TRL-9 photomicrographs and 25µm in the isotype control.

4.2.5.2.2 Cytokine responses after TLR9 stimulation of primary type II alveolar epithelial cells

After observing that primary type II alveolar epithelial cells expressed TLR9 strongly in their cytoplasm, I decided to characterise their responses to stimulation with CpG-C DNA. Cells from seven patients who underwent lung resection were used for these experiments. Patient demographics are shown in Table 4.2

Patient	*Age (Yr)*	*Sex*	*Lobe resected*	*Final diagnosis*	*Smoker*
5	59	F	LU	Squamous cell Ca	Y
6	57	M	LL	Non small cell lung Ca	Y
7	70	F	RU	Bronchogenic Ca	Y
8	63	F	L*	Single lung metastasis from malignant melanoma	N
9	64	F	L*	Squamous cell Ca	Y
11	59	F	RU	Single lung metastasis from rectal Ca	N
12	60	F	M	Non small cell lung Ca	Y

Table 4.2 Characteristics of patients from whom primary type II alveolar epithelial cells were isolated.
RL, right lower lobe; LL, left lower lobe; RU, right upper lobe; M, right middle lobe; *Pneumonectomy; N/A, non applicable.

The results show a significant increase in the production of IL-8 after stimulation with CpG-C DNA (p=0.03). IL-1β secretion displayed a modest increase. There were variable results for IL-6, IL-10, TNF-α and IL-12p70 (Figure 4.8)

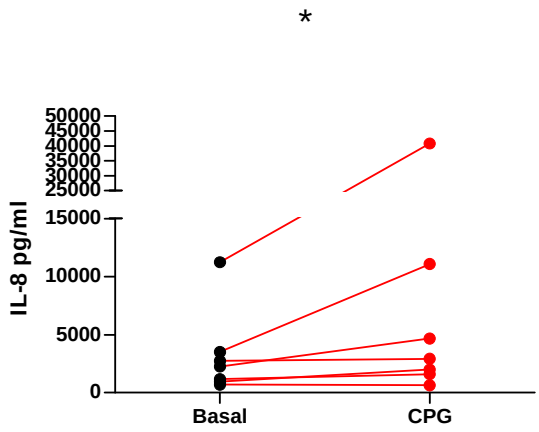


Figure 4.8 IL-8 responses by primary human type II alveolar epithelial cells after stimulation of TLR9.
Cells were seeded in 6 well plates pre-coated with bovine type I collagen. After confluence, cells were washed and stimulated with 1μM CpG-C DNA for 24 hours.
Data are presented as individual data points.
Statistical analysis was performed using the Wilcoxon matched pairs test to compare results for paired samples before and after stimulation using GraphPad Prism software.
Statistical significance (*) was determined when $p<0.05$.

4.2.5.3 Comparison of the cytokine response to TLR9 stimulation in primary human nasal and type II alveolar epithelial cells

The cytokine response varied greatly between cells of the upper and lower respiratory tract after stimulation of TLR9 with CpG-C DNA. Type II alveolar epithelial cells appeared more responsive to stimulation with CpG-C DNA given

the significant increase in the secretion of IL-8 in supernatant and a trend toward the increased secretion of IL-1β. The nasal epithelial cells appeared refractive to stimulation and results varied greatly among subjects, despite showing a strong stain for TLR-9 in their cytoplasm. For a summary of the cytokine responses please refer to Table 4.3.

These results suggest that cells from the lower respiratory tract behave in a more pro-inflammatory pattern after TLR9 stimulation, whereas cells from the upper respiratory tract appear more tolerant to the presence of bacterial DNA.

Cytokine	Primary nasal epithelial cells Basal (pg/ml)	Primary nasal epithelial cells CpG (pg/ml)	p value	Primary type II alveolar epithelial cells Basal (pg/ml)	Primary type II alveolar epithelial cells CpG (pg/ml)	p value
IL-8	282 (193-1004)	120 (11.8-2531)	1	2273 (707-11226)	2919 (636 – 40775)	*0.0313
IL-6	26.2 (13.7-167.8)	20.2 (4.7-535)	1	236.3 (8.3-1276)	228.3 (12.6–803)	0.57
IL-1β	7.1 (0-9.5)	4 (0-10.5)	1	5.2 (2.8-8.1)	7.5 (1.7-11)	0.078
TNF-α	10.3 (1.7-14.9)	5.4 (0–17.5)	0.18	10 (3.6-21.2)	7 (0–15.7)	0.81
IL-10	10.4 (4-18.7)	6.2 (0–21.1)	0.43	15.2 (2.6-1276)	20.2 (0–803)	0.37
IL-12p70	12.6 (3.6-19.8)	2.1 (0–26.7)	0.43	8 (5.4-19.7)	12 (2.7-28.6)	0.078

Table 4.3 Comparison of cytokine responses between human primary nasal epithelial cells and primary human type II alveolar epithelial cells before and after stimulation of TLR9

Data are expressed as median (range) for 5 and 7 subjects for primary nasal and type II alveolar epithelial cells respectively.
Statistical analysis was performed using the Wilcoxon matched pairs test to compare results for paired samples after stimulation using GraphPad Prism software.
Statistical significance (*) was determined when $p<0.05$.

After demonstrating that primary epithelial cells from the upper and the lower respiratory tract express TLRs, I wanted to assess whether these cells act under negative regulation mechanisms such as the ones that have been identified in the gastrointestinal tract. For this, and in view of previous evidence of a recently identified protein called toll-interacting protein (tollip), I went on to try to identify if this protein could be expressed in the respiratory tract.

4.3 Role of Toll-interacting protein (tollip) in the respiratory tract

The respiratory tract is continuously challenged by the presence of micro-organisms and inorganic particulate matter that enters during breathing. Mounting an inflammatory response every time that the epithelium is challenged would have deleterious consequences for the host, therefore negative regulatory mechanisms should be in place to avoid the excessive reaction to foreign particulates as well as invading micro-organisms. The gastrointestinal tract is an excellent example of this, since its large surface area is covered by multiple types of bacteria that live within without causing alterations in homeostasis. Furthermore, when the tolerance mechanisms are disregulated, pathology ensues (Shibolet, O., and Podolsky, D., 2007). One of the negative regulators firstly identified in the gastrointestinal tract is tollip, which appears to decrease inflammatory responses in colonic epithelium by inhibiting the transcription factor NF-κB (Otte, J., et al, 2004 and Melmed, G., et al, 2003).

Since tollip's actions could also exert an important effect in the respiratory tract, my aim was to assess if this protein is expressed in cell lines representative of the respiratory tract and then to characterise its responses in human primary epithelial cells.

4.3.1 Identification of tollip in the Human Colon Carcinoma cell line T84

4.3.1.1 Detection of tollip messenger RNA

In view of previous published data obtained in intestinal epithelial cells (Melmed, G., et al, 2003 and Otte, J., et al, 2004) I used the human colon carcinoma T84 cell line as the starting point to produce primers against *tollip*, in order to use it as a positive control. Two sets of primers were used to achieve the detection of *tollip* mRNA in the cell line T84. An in-house set of primers was designed with the help of Dr. Thomas Wilkinson using the software Primer3®: the forward primer OM1:TACAGGCAAAGTTGGCCAA, binds a region of the *tollip* gene between the second and third exon, the reverse primer OM2:AAGCAGCGCGTAGGACAT, binds a region between the fourth and fifth exons. The expected product size was 347 base pairs.

For the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) housekeeping gene PCR was performed for 20 cycles. Primers used were designed using Primer3 software; forward primer: 5'-CGAGATCCCTCCAAAATCAA-3' and

reverse primer: 5'-TGCTGTAGCCAAATTCGTTG-3'. The product size expected was 727 base pairs.

A second PCR reaction was performed using primers published by Otte, J., et al, 2004. The expected product size was 74 bp. The PCR conditions are described in the Materials and Methods page 73 section 2.9.

I identified transcripts for tollip in the GI cell line T84. (Figure 4.9 and Figure 4.10).

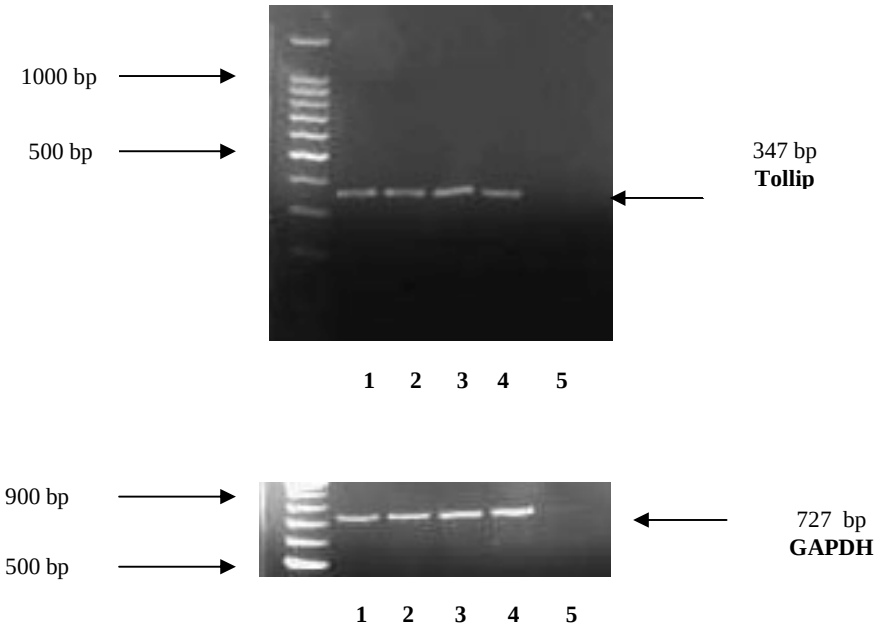


Figure 4.9 Expression of *tollip* mRNA analysed by RT-PCR in the human colonic cell line T84 using primers OM1 and OM2.
T84 cells were plated at two different cell densities: 5 x 10^5 per well (lanes 1,2); 2 x 10^6, (lanes 3,4) and negative control without the reverse transcriptase (lane 5).
GAPDH was used as a housekeeping gene.

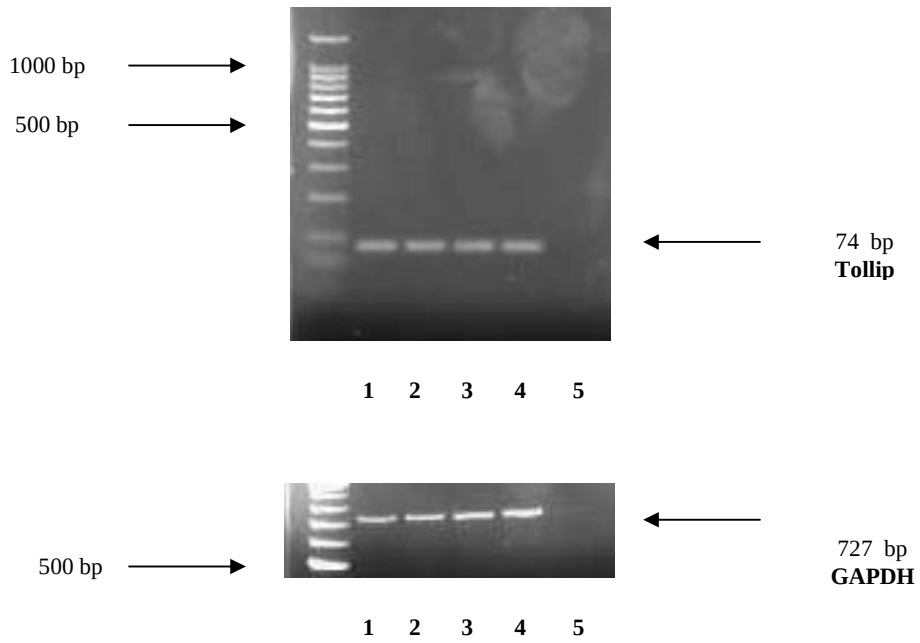


Figure 4.10 Expression of *tollip* mRNA analysed by RT-PCR in the human colonic cell line T84 using primers described by Otte et al.
T84 cells were plated at two different cell densities: 5 x 10^5 per well (lanes 1,2); 2 x 10^6, (lanes 3,4) and negative control without the reverse transcriptase (lane 5).
GAPDH was used as a housekeeping gene.

4.3.1.2 Detection of tollip protein in the Human Colon Carcinoma cell line T84

Once the transcripts for tollip in the human colon carcinoma cell line T84 were found, I designed an in-house direct ELISA with the aim to detect tollip protein as described in the Materials and Methods page 69 section 2.8. High intracellular levels of tollip were found in cell lysates of the T84 cell line. Tollip was not detected in cell culture media or in cell-free supernatants. (Figure 4.11)

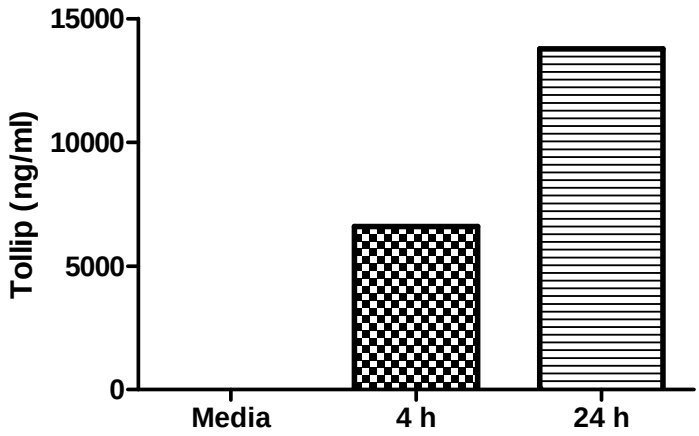


Figure 4.11 Tollip levels in the human colon carcinoma cell line T84.
T84 cells were seeded at 2 x 10^6 cells/ml in 6 well plates. Once confluent, cells were lysed and cell lysates were assayed for tollip by direct ELISA.

4.3.2 Detection of tollip in the cell line RPMI 2650

4.3.2.1 Immunocytochemistry

Using confocal microscopy it was possible to find tollip in the nasal epithelial cell line RPMI 2650. The cells stained diffusely and strongly in the cytoplasm. In some cells the nucleolus also appeared to stain positively for tollip. (Figure 4.12)

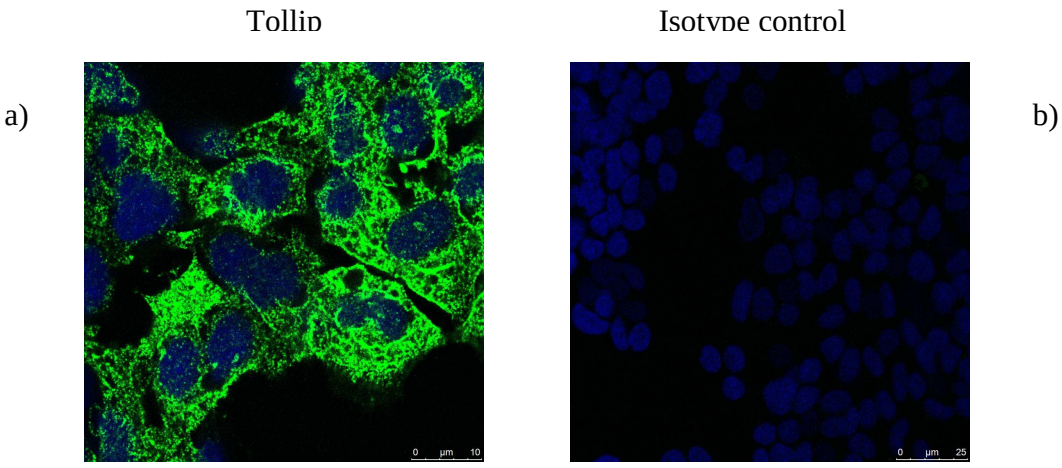


Figure 4.12 Detection of tollip in the nasal epithelial cell line RPMI 2650.
Cells were seeded onto coverslips, fixed with methanol, blocked with 2% goat serum and incubated with a polyclonal rabbit antibody against tollip. Nuclei were stained with DAPI (blue). Secondary antibody was anti-rabbit IgG conjugated with Alexa 488 (green).
Images were analysed using confocal microscopy. a)tollip b)Isotype control.
Scale bar equals 10μm to appreciate the staining in the cells, the isotype control scale bar equals 25μm.
Strong cytoplasmic stain can be observed in the cells in panel a.

4.3.2.2 m-RNA tollip levels during infection with *S. aureus* strain Newman

I decided to ask whether tollip m-RNA species could be found in the nasal cell line RPMI 2650 and if expression was affected after infection with *S. aureus* strain Newman. m-RNA transcripts for *tollip* were found in the RPMI 2650 cell line, which seems to express constitutively high levels. The levels did not seem to be affected after infection with two different doses of live *S. aureus*, nor were they affected after the cells were incubated with the same dose of ultraviolet light-killed bacteria after 4 hours of stimulation. The T84 cell line was used as the positive control for this experiment and it was interesting to observe

decreased intensity in the expression of *tollip* transcripts in this cell line in comparison to the RPMI 2650 cell line (Figure 4.13).

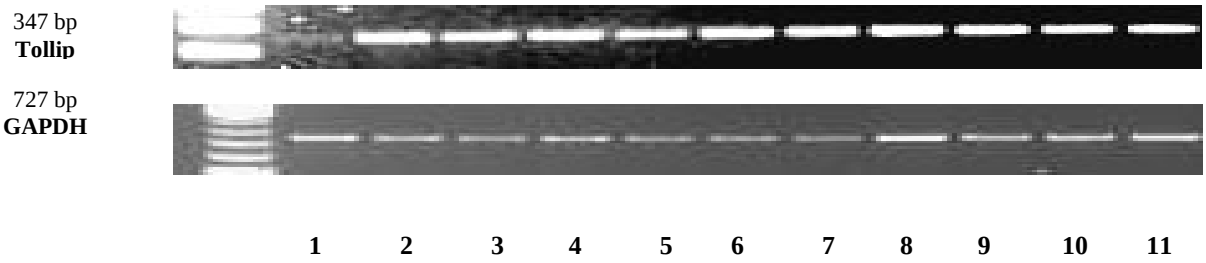


Figure 4.13 Expression of *tollip* mRNA in the human nasal epithelial cell line RPMI 2650 before and after 4 hours of infection with *S. aureus* strain Newman.
RPMI 2650 cells were plated at 1 x 10^6 per well. Once the cells achieved 90% confluence, they were infected as described previously in the Materials and Methods section. RNA extraction was performed followed by RT-PCR using the primers OM1 and OM2
Lane 1) positive control for *tollip* from cell line T84; 2,3)unstimulated RPMI 2650 cells 4,5) RPMI 2650 cells infected with *Staphylococcus aureus* strain Newman at a concentration of 1.1 x 10^5cfu/ml 6,7)RPMI 2650 cells infected with *S. aureus* at 1.6 x 10^5 8,9)Cells exposed to UV-killed *S. aureus* at a concentration of 1.1 x 10^5cfu/ml 10,11)cells exposed to υ killed *S. aureus* at a concentration of 1.6 x 10^5cfu/ml.
Transcripts for *GAPDH* were used as the housekeeping gene

These findings suggest that *tollip* m-RNA expression is constitutive in RPMI 2650 cells and it does not seem to be affected by infection with *Staphylococcus aureus* at an early stage (4h).

It was then relevant to assess whether *tollip* m-RNA transcripts would be affected at a later point during infection. Therefore an experiment was designed to assess this at 24 hours. The results confirmed that RPMI 2650 cells express

constitutive levels of *tollip* m-RNA at 24 hours. However, after exposure to live *S. aureus* strain Newman, there were no bands either in the lanes that contained the gene of interest or in the lanes concerning the house keeping gene *GAPDH*. In contrast, the wells in which the cells were exposed to *S. aureus* killed by ultraviolet light remained unchanged. (Figure 4.14)

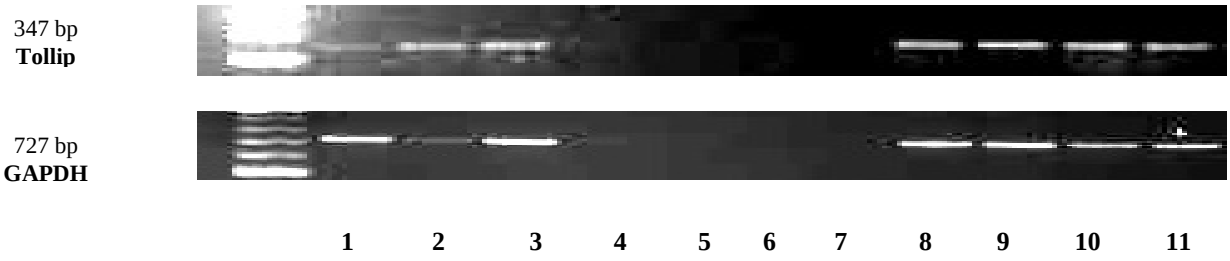


Figure 4.14 Expression of *tollip* mRNA in the human nasal epithelial cell line RPMI 2650 before and after 24 hours infection with *S. aureus* strain Newman.
RPMI 2650 cells were plated at 1 x 10^6 per well. Once the cells achieved 90% confluence, they were infected as described previously. RNA extraction was performed followed by RT-PCR using the primers OM1 and OM2.
Lane 1) positive control for *tollip*, cell line T84; 2,3)unstimulated RPMI 2650 cells; 4,5) RPMI 2650 cells infected with *S. aureus* at a concentration of 1.1 x 10^5cfu/ml; 6,7)Cells infected with *S. aureus* at 1.6 x 10^5cfu/ml; 8,9)Cells exposed to killed *S. aureus* at 1.1 x 10^5 cfu/ml 10,11)cells exposed to killed *S. aureus* at 1.62 x 10^5 cfu/ml.
GAPDH was used as the housekeeping gene.

The absence of bands suggests cellular death caused by the infection with *S. aureus* at 24 hours, this observation was supported morphologically, and therefore, that time point had to be avoided.

4.3.2.3 Role of tollip during infection with a MRSA strain

After it was noted that a methicillin-sensitive strain of *S. aureus* had no effect on the transcription of *tollip* at 4 hours, I wanted to assess whether there was a different response after infection with a methicillin-resistant strain of *S. aureus*. The MRSA strain 252 was a kind gift from Dr. Ross Fitzgerald from the Centre for Infectious Diseases (CID), University of Edinburgh. This strain was used for these experiments (Figure 4.15).

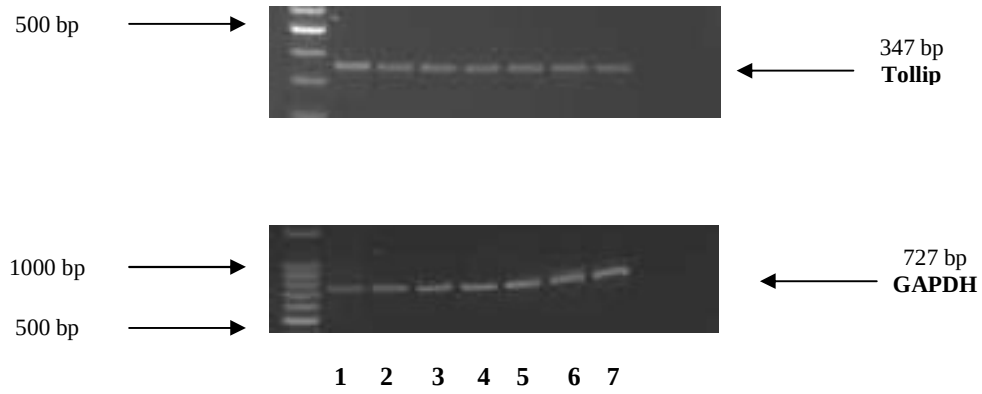


Figure 4.15 Expression of tollip mRNA in the nasal epithelial cell line RPMI 2650 before and after infection with MRSA strain 252 for four hours.
RPMI 2650 cells were plated at 1 x 10^6 per well. Once 90% confluence was achieved, they were infected as described previously. RNA extraction was followed by RT-PCR using primers OM1 and OM2.
Lanes: 1) positive control for *tollip* from cell line T84, 2,3) unstimulated cells, 4,5) cells infected with MRSA 252 at a concentration of 1.1 x 10^5cfu/ml and 6,7) cells infected with MRSA 252 at 1.6 x 10^5 cfu/ml.
GAPDH was used as a housekeeping gene.

The results of PCR showed a similar result to those using the MSSA strain. *Tollip* constitutive levels were not changed after infection with a MRSA strain.

4.3.2.4 Role of quantitative PCR for tollip during *S. aureus* infection

Since the conventional nested PCR did not provide clear data about regulation of tollip during *S. aureus* infection, I decided to use a quantitative method to assess the changes at the transcription level. QPCR primers and probe were designed with the assistance of Dr. Thomas Wilkinson using the software Primer3®: forward primer: 5'-TGGAATAAGGTCATCCACTG-3', reverse primer: 5'-AGAAGGCTCTCTCATCGAA-3', and probe: 5'-CCCAGGCGTGGACTCTTTCTA-3'.

Primers and probe were also designed for the housekeeping gene GAPDH using the same software. Forward primer: 5'-GAAGGTGAAGGTCGGAGTC-3', reverse primer: 5'-GAAGATGGTGATGGGATTTC-3' and probe: 5'-CAAGCTTCCCGTTCTCAGCC-3'. Primers were tested and dissociation curves were performed to assess specificity and to rule out the presence of primer dimmers.

QPCR was performed in the samples after a 4 hour infection of the cell line RPMI 2650 with *S. aureus*. It was possible to observe up-regulation in the transcripts for *tollip*, although when assessing the response in the housekeeping genes the results were contradictory. Whereas *GAPDH* appeared to be up-regulated upon infection, *18S* appeared to be down-regulated as a result of *S.*

aureus infection making a relative quantification of the tollip results difficult (Figure 4.16).

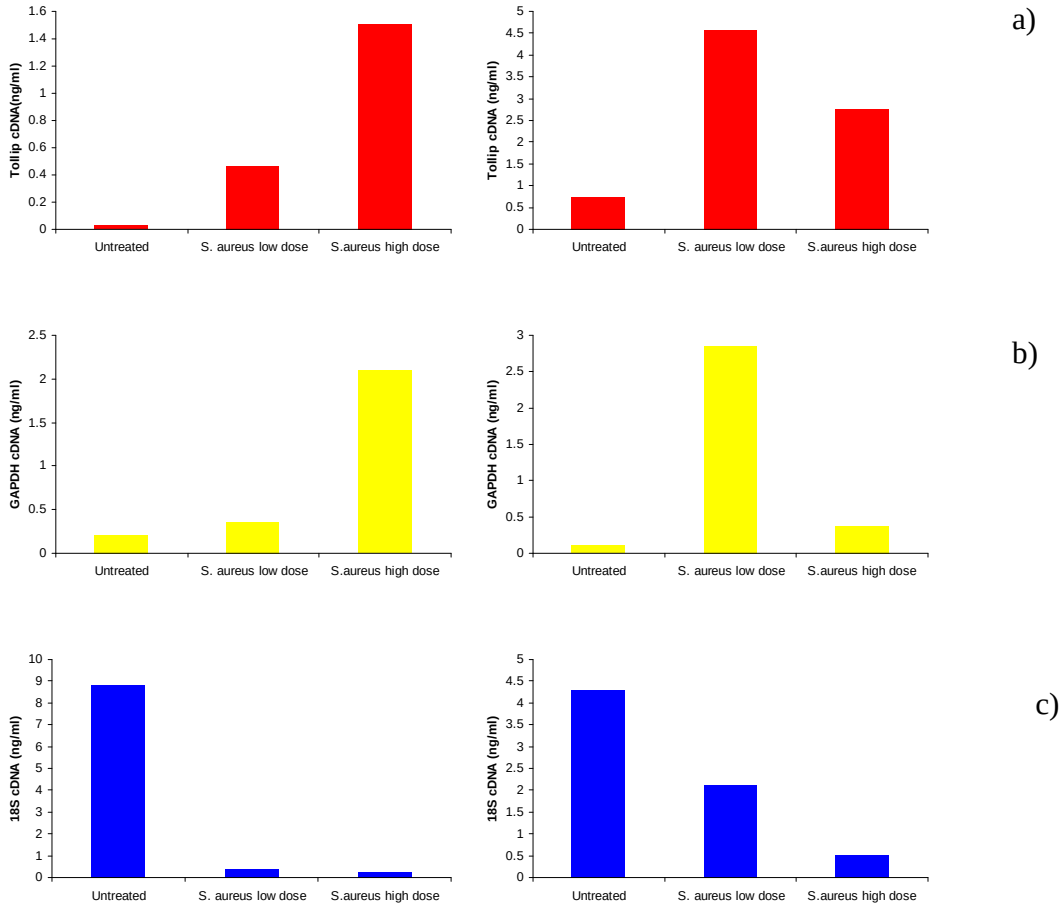


Figure 4.16 Expression of *tollip, GAPDH* and *18S* mRNA in the human nasal epithelial cell line RPMI 2650 before and after 4 hours infection with *S. aureus* strain Newman.
RPMI 2650 cells were plated at 1 x 10^6 per well. Once the cells achieved 90% confluence, they were infected as described previously in the Materials and Methods section. RNA extraction was performed followed by a)*tollip* QPCR using the primers and probe as described above, b)*GAPDH* and c)*18S* were used as house keeping genes.
Results are presented as means for two separate experiments.

The lack of correlation between the gene of interest and the housekeeping genes probably indicates that the cells are overwhelmed by the infection with multiple cellular processes occurring simultaneously. This makes the model a very difficult one for establishing a mechanism.

4.3.2.5 Tollip protein levels in the cell line RPMI 2650

Due to the difficulties trying to establish an *in-vitro* live model of infection, I decided to move onto a model in which epithelial cells were in contact with bacterial ligands in order to try to establish the behaviour after stimulation with single proteins. RPMI 2650 cells were plated, allowed to reach confluence and then left untreated or stimulated with TLR ligands such as ultrapure *Pseudomonas aeruginosa* LPS, *S. aureus* PGN and LTA, and the cytokine TNF-α. Tollip appeared to be an abundant protein in this cell line with median baseline intracellular levels of 5922ng/mg protein (range: 1635 – 26067). These levels did not seem to increase after stimulation with *P. aeruginosa* LPS or *S. aureus* PGN or LTA. Stimulation with the pro-inflammatory cytokine TNF-α was not associated with significant changes in tollip protein levels.

4.3.3 Identification of tollip in the cell line A549

4.3.3.1 Immunocytochemistry

Using confocal microscopy it was possible to detect tollip protein in the human type II alveolar cell line A549. The stain was cytoplasmic and varied in intensity.

Some cells stained strongly whereas others showed no stain. In some cells it was possible to observe nuclear staining for tollip (Figure 4.17).

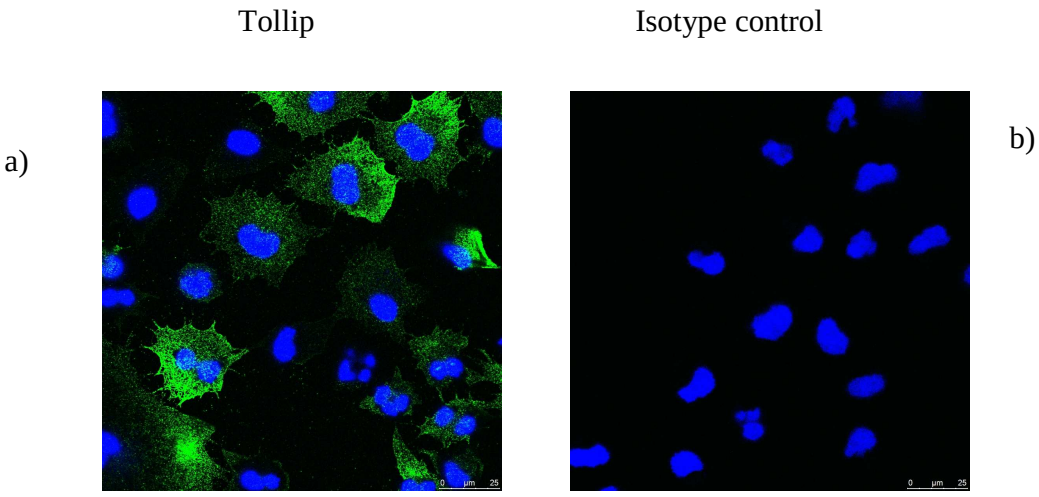


Figure 4.17 Detection of tollip in the alveolar epithelial cell line A549.
Cells were seeded onto coverslips, fixed with methanol, blocked with 2% goat serum and incubated with a polyclonal rabbit antibody against a)tollip, b)Isotype control. Nuclei were stained with DAPI (blue). Seconary antibody was anti-rabbit IgG conjugated withAlexa 488 (green).
Images were analysed using confocal microscopy.
Strong cytoplasmic stain can be observed in the cells.
Scale bar equals 25µm.

4.3.3.2 m-RNA tollip levels during infection with *S. aureus* strain Newman

After the successful identification of tollip protein by immunocytochemistry, m-RNA transcripts for *tollip* were found in the cell line A549. Expression seemed to be constitutive and not influenced after infection with *S. aureus* strain Newman. *GAPDH* was used as the housekeeping gene (Figure 4.18).

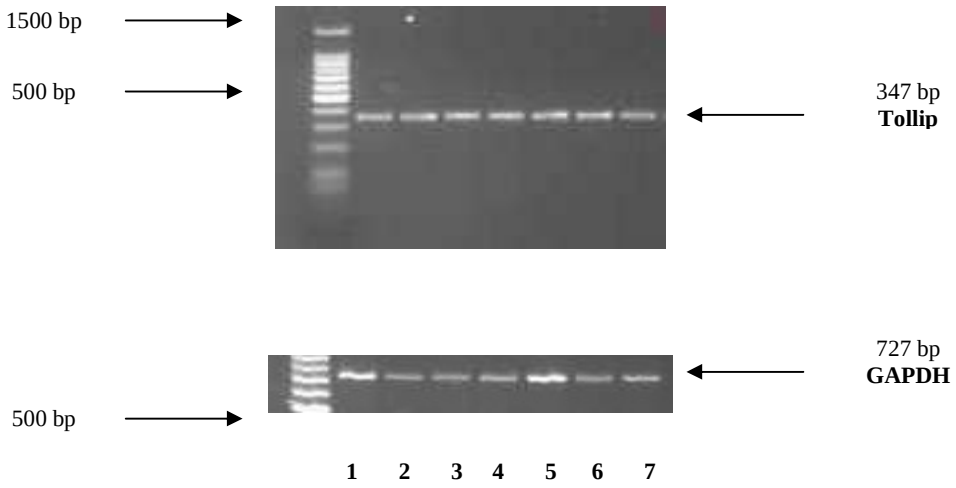


Figure 4.18 Expression of *tollip* mRNA in the human alveolar epithelial cell line A549 before and after 4 hours of infection with *S. aureus* strain Newman.
A549 cells were plated at 1 x 10^6 per well. Once 90% confluence was achieved, they were infected as described previously. RNA extraction was followed by RT-PCR using primers OM1 and OM2.
Lanes: 1) positive control for *tollip* from cell line T84, 2,3) unstimulated cells, 4,5) cells infected with *S.aureus* at a concentration of 1.1 x 10^5cfu/ml and 6,7) cells infected with *S. aureus* strain Newman at 1.6 x 10^5 cfu/ml.
GAPDH was used as a housekeeping gene.

A549 cells were plated at 1 x 10^6 per well and then infected with two different doses of *S. aureus* strain Newman as described in the Materials and Methods section 2.7, page 68, and RT-PCR was performed. The findings were similar to those for RPMI 2650 cells, i.e. *tollip* m-RNA transcripts were found in the A549 cell line but there was not a significant increase in the quantity of the transcripts during bacterial infection when compared to transcripts for *GAPDH*.

4.3.3.3 Tollip protein levels in the cell line A549

Median constitutive tollip levels in the human epithelial cell line A549 were 2886ng/mg of protein (range: 444 – 30435). There were modest increases in the intracellular levels after stimulation of the cells with LPS from *P. aeruginosa* and PGN from *S. aureus*, although these did not achieve statistical significance. TNF-α stimulation did not have any effects on intracellular levels of tollip.

4.4 Identification of Tollip in primary cells of the respiratory tract

After the demonstration of tollip in cell lines, I decided to assess if it would be possible to transfer the same techniques used onto primary human epithelial cells in order to confirm the presence of this protein in a more physiological model. For this cells from human volunteers were used.

4.4.1 Immunocytochemistry in nasal cells

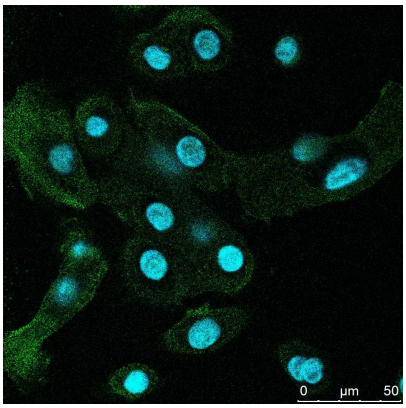
Primary human nasal epithelial cells were obtained from healthy volunteers and patients undergoing partial or total lobectomy due to lung neoplasias as described in Chapter 3.

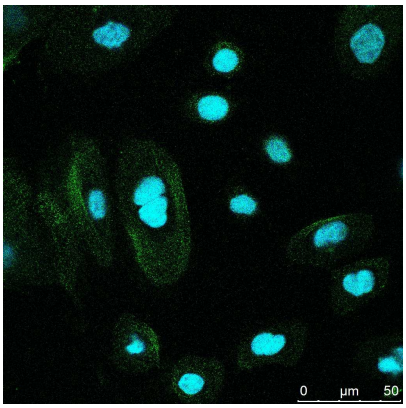
Using confocal microscopy it was possible to find tollip in the primary nasal epithelial cells, these cells stained in a different pattern when compared with their cancer cell line counterpart. There is evidence of punctate staining distributed diffusely around the cytoplasm, probably in the endosomal compartment, although markedly less strong than for the cell lines RPMI2650 or

A549 (see pages 184 and 192 respectively). The pattern of the stain appears to be similar to the observations made by Brissoni, B., et al, 2006 when they studied mouse embryonic fibroblasts (MEFs) and observed staining for tollip in the cytosol and suggested that this localisation is likely to be in late endosomes. More recently, Ciarrocchi, A., and coworkers, (2009) demonstrated in HeLa cells that tollip is found in small aggregates in the cytoplasm in a perinuclear position that could correspond to the Golgi apparatus and/or endosomes. In comparison to the cell line the primary nasal epithelial cells displayed a weaker nucleolar staining.

After demonstrating the evidence of tollip in primary nasal epithelial cells, I wanted to assess whether the location of this protein was altered in response to stimulation with TLR ligands. In relation to the effects of different stimuli on the staining pattern, it was interesting that the cells that received *S. aureus* PGN showed multiple aggregates of tollip protein around the cytoplasm. It would be interesting in the future to follow this observation further to ascertain if these aggregates are formed in early or late endosomes. There were no changes in the distribution of tollip in the cells stimulated with TNF-α, as expected, TNF-α acts through a different pathways. (Figure 4.19)

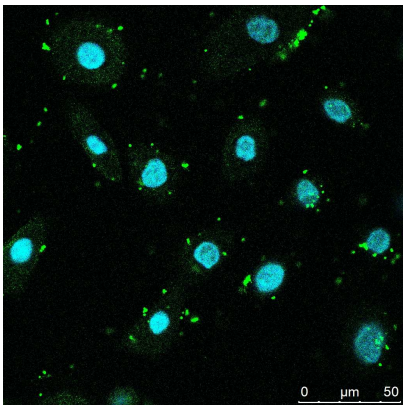
a) Untreated

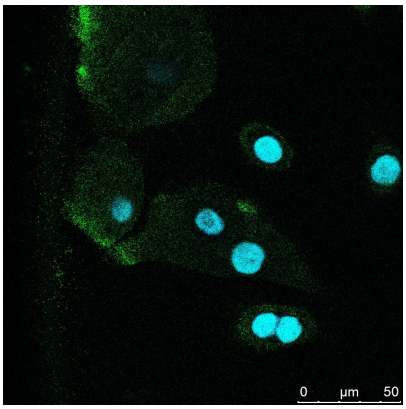




b) LPS

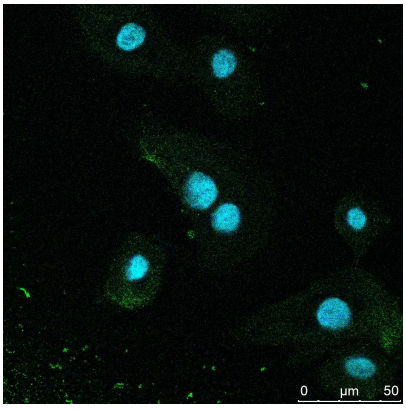
c) PGN

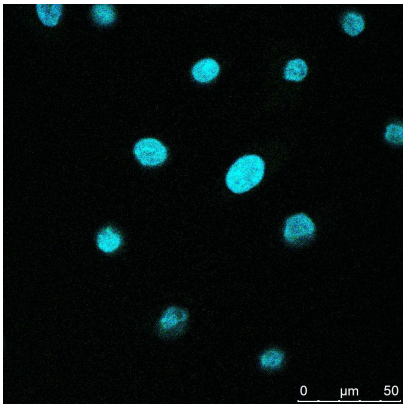




d) LTA

e) TNF





f) Isotype control

Figure 4.19 Detection of tollip in primary human nasal epithelial cells.
Cells were seeded onto coverslips for 24 hours and then stimulated with TLR ligands or TNF-α: a) untreated, b) LPS, c) PGN, d)LTA, e) TNF, f) isotype control for 24 hours. They were then fixed with methanol, blocked with 2% goat serum and incubated with a rabbit polyclonal antibody against tollip. Nuclei were stained with DAPI (blue). Secondary antibody was anti-rabbit IgG conjugated with Alexa 488 (green).
Images were analysed using confocal microscopy.
Diffuse cytoplasmic stain can be observed in the cells. In the cells exposed to petptidoglycan there is evidence of deposits of tollip around the cytoplasm and in the nucleus. There is no evidence of change in the appearance of the deposits in the cells treated with TNF-α.
Scale bar equals 50μm.
Results presented are of one representative experiment of four.

4.4.1.1 Role of tollip m-RNA transcripts in nasal epithelial cells

As seen previously I identified tollip protein in human primary nasal epithelial cells (Figure 4.19 above). QPCR was performed on 5 sets of primary nasal epithelial samples from the patients illustrated in Table 4.3. RNA was extracted after lysis of the cells and real-time PCR was performed. *Tollip* CT values were normalised to *18S* which was used as the housekeeping gene; results are given as a relative value of the expression of these two genes using the standard curve method.

Subject	Age	Sex	Smoker
Patient 3	66	M	Y
Patient 6	57	M	Y
Patient 9	64	F	Y
Patient 20	57	M	Y
Patient 23	69	F	Y

Table 4.3 Characteristics of the five patients from whom primary nasal epithelial cells were isolated and PCR was performed

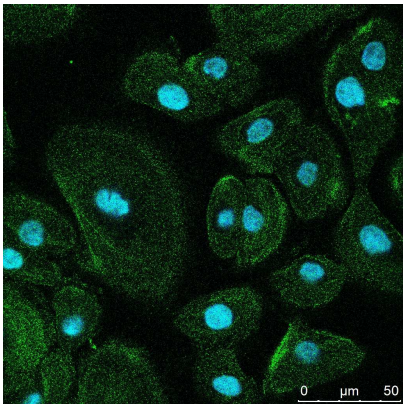
The results show great variability in the tollip m-RNA transcripts from the five different patients. Stimulation with LPS, PGN or LTA was not associated with an increase in tollip transcription. Stimulation with TNF-α was not associated with an up-regulation of this gene either, as expected.

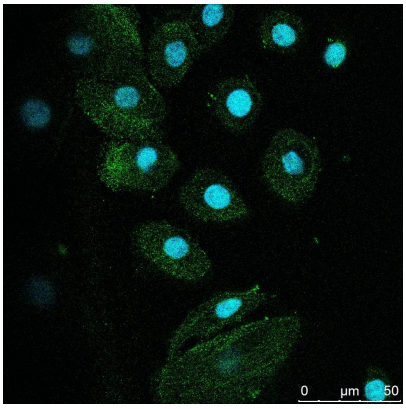
4.4.2 Immunocytochemistry in bronchial epithelial cells

Using confocal microscopy it was possible to find tollip in the primary bronchial epithelial cells from a patient. These cells stained in a similar way in comparison with their primary nasal epithelial cell counterparts. There is evidence of a diffuse punctate staining in the cytoplasm, nucleolar staining is weak. This pattern appears to be consistent with my previous observations made in primary nasal epithelial cells.

In relation to the effects of different stimuli on the staining pattern, the cells that received PGN from *S. aureus* showed multiple aggregates of tollip protein around the cytoplasm, and more particularly in this case toward the cellular membrane as well as some aggregates in the nucleus. Unfortunately due to technical difficulties it was not possible to isolate and culture a larger number of cells from different subjects and therefore, there are limitations to these findings. It would be interesting in the future to follow this observation further. There were no changes in the distribution of tollip in the cells stimulated with TNF-α as expected (Figure 4.20).

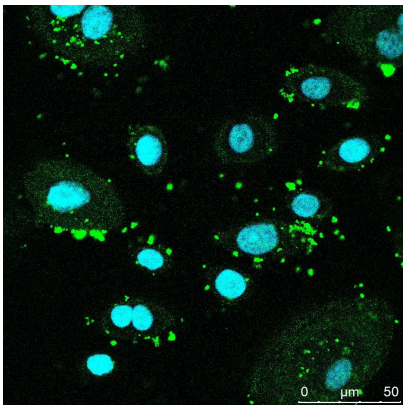
a) Untreated

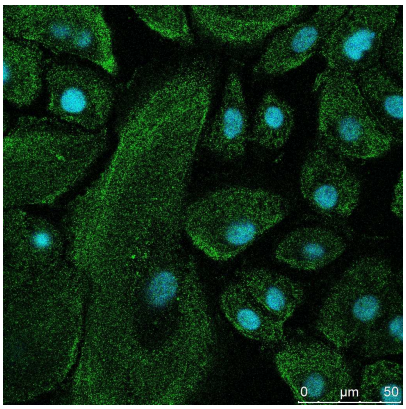




b) LPS

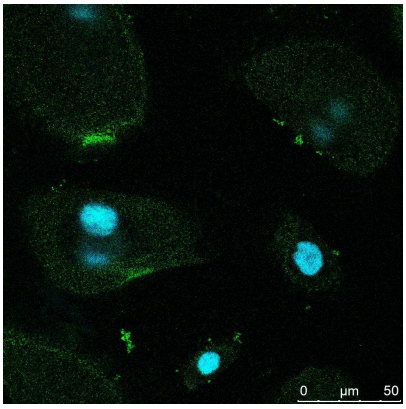
c) PGN

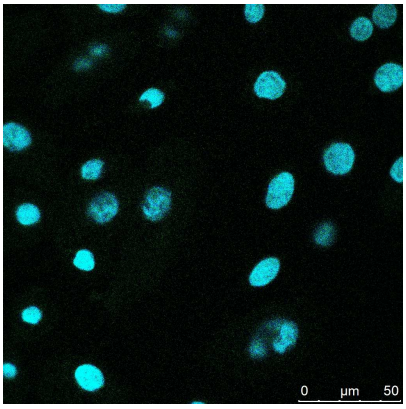




d) LTA

e) TNF





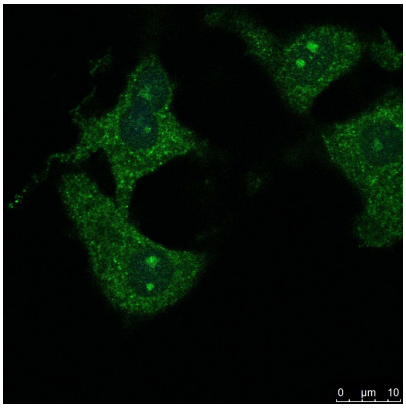
f) Isotype control

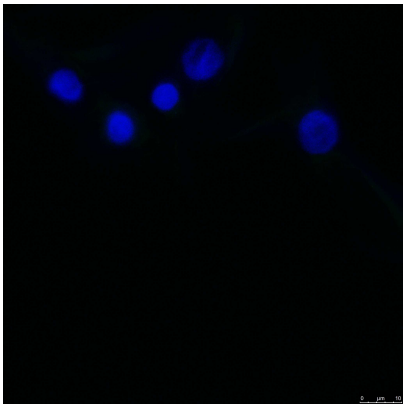
Figure 4.20 Detection of tollip in primary human bronchial epithelial cells.
Cells were seeded onto coverslips for 24 hours and then stimulated with TLR ligands as follows: a) untreated, b) LPS, c) PGN, d) LTA, e) TNF, f) isotype control for further 24 hours. They were then fixed with methanol, blocked with 2% goat serum and incubated with a rabbit polyclonal antibody against tollip. Nuclei were stained with DAPI (blue). Secondary antibody was anti-rabbit IgG conjugated with Alexa 488 (green).
Images were analysed using confocal microscopy.
Scale bar equals 50µm. Results presented are for one experiment.

4.4.3 Immunocytochemistry in primary type II alveolar epithelial cells

Following the demonstration of tollip in cells from the upper respiratory tract and the airway, I wanted to establish whether tollip was expressed in primary type II alveolar epithelial cells and to determine any differences. I prepared the conditions necessary to perform immunocytochemistry. Using confocal microscopy, I was able to find tollip in primary type II alveolar epithelial cells from a patient. These cells displayed a stronger cytoplasmic stain which, in comparison to the nasal and bronchial cells, appears more concentrated in the perinuclear region. It was also possible to observe a marked positive nucleolar staining (Figure 4.21).

a) Tollip





b) Isotype control

Figure 4.21 Detection of tollip in primary type II alveolar human epithelial cells.
Cells were seeded onto coverslips pre-coated with bovine type I collagen. They were then fixed with 4% paraformaldehyde, blocked with 2% goat serum and incubated with a polyclonal rabbit antibody against tollip. Nuclei were stained with DAPI (blue). Secondary antibody was anti-rabbit IgG Alexa 488 (green).
Images were analysed using confocal microscopy.
Strong diffuse cytoplasmic stain can be observed in the cells and a very strong nuclear stain can also be observed.
Scale bar equals 10µm.

4.4.3.1 Role of Tollip m-RNA in type II alveolar epithelial cells

As seen previously I identified tollip protein in human primary type II alveolar epithelial cells (Figure 4.21 above), although due to the technical difficulties involved in the isolation of these cells and the variable numbers obtained from different sized lung resection specimens, it was not possible to perform protein extraction for all of the samples from the different patients. It was however possible to extract RNA after lysis and to perform real-time PCR. QPCR was performed on 6 lung tissue samples from the patients illustrated in Table 4.4. *Tollip* CT values were normalised to *18S* which was used as the housekeeping gene. Results are given as a relative value of the expression of these two genes using the standard curve method.

Patient	*Age (Yr)*	*Sex*	*Lobe resected*	*Final diagnosis*	*Smoker*
5	59	F	LU	Squamous cell Ca	Y
6	57	M	LL	Non small cell lung Ca	Y
7	70	F	RU	Bronchogenic Ca	Y
8	63	F	L*	Single lung metastasis from malignant melanoma	N
9	64	F	L*	Squamous cell Ca	Y
12	60	F	M	Non small cell lung Ca	Y

Table 4.4 Patients from whom QPCR for *tollip* was performed.
RL, right lower lobe; LL, left lower lobe; RU, right upper lobe; M, right middle lobe; *pneumonectomy; N/A, non applicable.

The results suggest that tollip in the lower respiratory tract is up-regulated after stimulation with *P. aeruginosa* LPS. PGN and LTA from *S. aureus* also demonstrated a trend toward up-regulation of this gene although there is higher variability among subjects in response to these stimulants and the differences are not statistically significant, possibly due to the small number of samples analysed. Taken together these results illustrate the similar behaviour of this protein in a completely different compartment in comparison to the gastrointestinal tract. There was not a significant change in *tollip* m-RNA levels after stimulation of primary type II alveolar epithelial cells with the pro-inflammatory cytokine TNF-α as expected.

4.4.4 Comparison of tollip response between the upper and the lower respiratory tract.

Following the identification of tollip m-RNA transcripts in cells of the upper and lower respiratory tract, the difference between reaction of these cells to challenge by bacterial ligands was analysed. It is well established that nasal colonisation of pathogens happens asymptomatically, whereas the presence of the very same organism in the lower respiratory tract is associated with a severe inflammatory response. The results show that constitutive m-RNA levels of tollip are significantly higher in nasal epithelial cells in comparison to their type II alveolar epithelial counterparts. However, both compartments tend to behave in a different

manner after challenge with bacterial ligands such as LPS, PGN or LTA. While it seems that LPS is associated with a more profound effect in the lung epithelial cells, there were no significant changes in the response of these cells after stimulation with PGN and LTA (Figure 4.22).

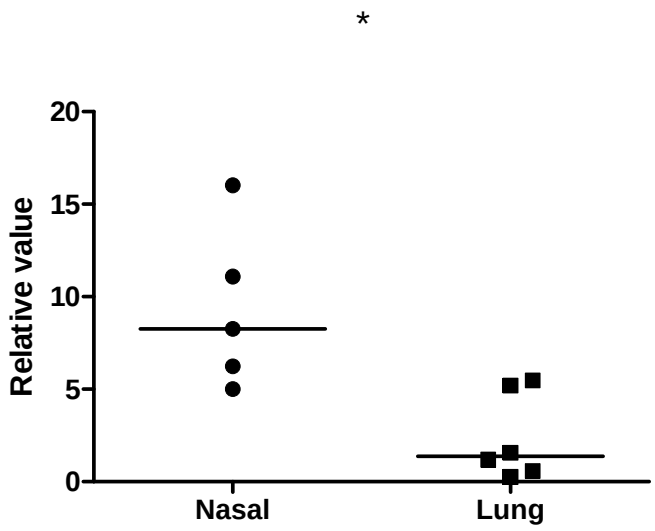


Figure 4.22 Comparison of tollip m-RNA transcripts in primary human nasal epithelial cells and primary human type II alveolar epithelial cells.
Human primary nasal epithelial cells were seeded onto 6 well plates and human primary type II alveolar epithelial cells were seeded onto collagen type I-pre-coated 6 well plates.
After achieving confluence, cells were washed and lysed, RNA was extracted and QPCR performed as described in the Materials and Methods section.
Data was analysed using the standard curve method and is presented as individual data points and median (line).
Statistical analysis was performed using the Mann-Whitney test for non parametric data.
Statistical significance (*) was determined when $p<0.05$.

4.5 Discussion

Primary human nasal epithelial cells express TLRs (Vandermeer, J., et al, 2004; Wang, J., et al, 2007 and Lane, A., et al. 2006). Although this fact is well established in the literature, I wanted to confirm the expression of these proteins in my system, using primary human monocytes as the positive control. Establishing differences in expression of these receptors among different subjects was out of the scope of the proposed research and therefore, there could be limitations to the data presented since TLR expression patterns/levels are subject to biological variation among individuals. The studies are consistent in finding high expression of TLR3 mRNA and low expression of TLR4 mRNA transcripts, and TLR2 mRNA expression was variable, although the limitation of these studies is that they were performed using specimens of nasal mucosa from patients with chronic rhinosinusitis. Furthermore, the study by Lane established differences in *TLR2* expression associated with this disease when they noted that the expression of this gene was increased in patients compared to healthy controls, although they did not establish a relationship with TLR2 protein levels in these patients.

In my study, using an immunocytochemistry approach I was able to find TLR1, TLR2, TLR4 and TLR9 in primary human nasal epithelial cells. I could not identify TLR6 in primary monocytes or primary nasal epithelial cells.

TLR1, displayed a diffuse cytoplasmic stain in primary nasal epithelial cells, whereas in monocytes it was expressed in the cytoplasmic membrane. Primary nasal epithelial cells also expressed TLR2 at a lower degree than primary human

monocytes. This finding coincides with the one from Wang and coworkers in 2007, in which although they could not detect significant expression of TLR2 protein, when looking into transcription, they detected low levels of *TLR2* mRNA transcripts. These findings are supported further by the observation from Mayer, A., et al (2006) when they studied bronchial epithelial cells and found low expression of *TLR2* mRNA which correlated in their studies to unresponsiveness to certain TLR2 ligands. They suggest that in airway epithelium low *TLR2* expression might play a role in limiting uncontrolled activation by inhaled bacteria.

In my experiments in primary human nasal epithelial cells, TLR4 appears predominantly as intracellular ring-like structures. This result is in accordance with the report by Guillot, L., et al, 2004, in which they found that in pulmonary epithelial cells and human primary bronchial epithelial cells, TLR4 localises intracellularly.

I was unable to identify TLR6 in primary human monocytes or in primary human nasal epithelial cells. This finding is consistent with the data reported by Nakao, Y., and colleagues, (2005) when they looked at the expression profiles of TLR1 and TLR6 and found that the expression varied within different subsets of cells from peripheral human blood. Further observations by Greene, C., et al, 2005 noted that when the expression of cell surface protein for TLRs was examined in the airway cell lines CFTE29o$^-$ and 16HBE14o$^-$, cell surface expression was detected for TLRs 1-5 and TLR9 but not TLR6 using laser scanning microscopy, although they were able to identify mRNA transcripts for this gene.

TLR9 has been shown to be essential for the recognition of bacterial DNA (Hemmi, H., et al, 2000). One of the main characteristics of bacterial genomes is that they contain a higher frequency of unmethylated deoxycytidyl-deoxyguanosine (CpG) dinucleotides (Krieg, A., et al, 1995 and Bauer, S., et al, 2001). Small oligodeoxynucleotides (ODN) with unmethylated CpG dinucleotides (CpG ODN) are able to mimic the immunostimulatory activity of bacterial DNA. It has been reported that the CpG backbone itself has immunostimulatory effects independent of TLR9 agonist activity (Jurk, M., et al, 2004). There are three different types of oligonucleotides. CpG class A (also called type D) ODN, are especially potent in activating human plasmacytoid dendritic cells (pDC) to produce high amounts of IFN-α. It has also been noted that this type of CpG is also a poor B cell stimulator. CpG class B, also called K-type, is a very potent Th1 inducer, has anti-tumour activity and stimulates strong B and NK cell activation and cytokine secretion. More recently, a third class of CpG (class C) has been characterised by Vollmer, J., and colleagues, (2004). They demonstrated that this class strongly stimulates B cells as well as type I IFN secretion in vitro on human peripheral blood mononuclear cells, and in vivo it also acts as a strong Th1 inducer. It is also efficient in enhancing expression of co-stimulatory molecules causing B cell production of IL-6 and IL-10 as well as stimulating strong B cell proliferation. It has also been demonstrated that this class of CpG induces IL-6 secretion in mouse splenocytes and also appears to induce more efficient IFN-γ secretion than the B-class CpG ODN in the same cells. CpG-C ODN also efficiently induces IFN-α-mediated NK cell lytic activity. CpG-C is also

associated with the down-regulation of CD14 and up-regulation of CD80 on monocytes, which characterises the transition from monocytes into myeloid DC and indicates that the C-class CpG ODN promotes the maturation of monocytes into functional DC that will be able to efficiently support antigen-specific cellular responses.

Jurk, M., et al (2004) demonstrated that CpG-C ODN strongly stimulated transcription of the chemokine IP-10 in human PBMC, as well as being associated with the induction of IL-18 mRNA up-regulation. IL-18 has been described as a cytokine that is able to directly induce IFN-γ production (Okamura, H., et al, 1995) and was also demonstrated to augment NK cell activity and to exhibit anti-tumour effects. They also reported that all three classes of CpG ODN were associated with down-regulation of TLR9 mRNA levels within 4 hours of incubation with human PBMC, with reconstitution to normal levels after 16 hours. C-class ODN appear to stimulate strong Th1-like responses in vitro on human PBMC, including type I and II interferon, IL-18 and IP-10 production and NK cell stimulation. They suggest that this type of ODN might prove to be useful to induce potent anti-tumour effects or long-lasting anti-viral activity in the therapy of chronic viral diseases. The study by Robbiani's group (2006) used the CpG-C ODN C274 in vivo and documented that it is a good activator of leukocytes (DCs and B cells) in the lymph nodes of macaques (both naïve and chronically infected with simian HIV (SHIV)). They also observed increase endogenous cytokine and chemokine activity in the lymph nodes injected, which could be important to help control virus replication within these tissues. After specific re-stimulation they

detected cellular activation and cytokine and chemokine responses and this finding places this group of CpG ODNs in the list of potential adjuvants to be used for improving the immunogenicity and protective efficacy of vaccines.

I chose to use the CpG-class C in my experiments because it is a potent immunostimulator, acts as an intermediate between classes A and B CpG ODNs, and it might have even broader local immune effects which are more likely to mimic the in-vivo situation in which dying bacteria within the airways will release a vast amount of bacterial DNA. In addition, there is evidence that it could be used as a potent adjuvant for the development of vaccines that could be nebulised to access the lower respiratory tract. So it could have a potential therapeutic effect. Moreover, although CpG class C ODN has been used to test responses from PBMC, dendritic cells and intranodally as described above, since there is to my knowledge no evidence of its responses in airway epithelial cells I thought that this would be a great opportunity to test its effects in primary human cells of the respiratory tract.

I found that TLR9 is strongly expressed in primary human nasal epithelial cells and the staining pattern was not affected by stimulation with TLR ligands. After stimulation with CpG-C ODN, the nasal epithelial cells displayed variable behaviour. IL-8 and IL-6 showed an increase in two subjects producing high levels, whereas in three subjects the levels tended to decrease. There was not a

clear association between TLR9 stimulation and the production of IL-1β, IL-10 and IL-12p70. TNF-α production appeared to be decreased in 4 subjects.

Primary lung epithelial cells expressed TLR9 strongly in their cytoplasm. However, it was not possible to assess the response after stimulation due to technical difficulties obtaining and culturing these cells. This would be an interesting area to assess in future research. The cytokine response in primary type II alveolar epithelial cells showed a significant increase in the production of IL-8 after CpG-C ODN stimulation and a modest increase in the production of IL-1β, although non statistically significant. Secretion of the other cytokines (IL-6, IL-10, TNF-α and IL-12p70) was unchanged.

The fact that TLR9 stimulation did not achieve a response in cells from the upper respiratory tract, whilst being more active in cells from the lower respiratory tract has also been documented previously in vivo by Albiger, B., et al (2006) when they challenged TLR9 deficient and wild type C57BL/6 mice intranasally with the *Steptococcus pneumoniae* strain TIGR4 and found no differences in bacterial counts in nasopharyngeal tracheal lavages from both types of mice. On the contrary, when examining BAL samples they observed that the $TLR9^{-/-}$ mice were less able to clear pneumonia given by the fact that 40% and 20% of wild type mice showed the persistence of bacteria in BAL samples at 8 and 12 hours post-infection respectively, in contrast to 70% and 60% of the mutant mice at the same

two time-points, indicating that TLR9$^{-/-}$ mice were significantly more susceptible to pneumococcal infection. They also found associated reduced survival and the development of bacteraemia. They did not observe a defect in the recruitment of immune cells to the lungs of the mutant mice, although they observed a decrease in the ability to phagocytose and kill pneumococci from the macrophages of the mutant mice. Furthermore, the effects of TLR9 deficiency on the susceptibility to develop pneumonia with *Legionella pneumophila* were shown by Bhan, U., et al, (2008) when they also showed increased mortality and reduced bacterial clearance in TLR9$^{-/-}$ mice in comparison to wild type BALB/c mice. They also noted a decrease in the internalisation of bacteria by alveolar macrophages from the mutant mice which was associated with a significantly decreased expression of iNOS mRNA by lung macrophages isolated from TLR9$^{-/-}$ mice, as in the absence of NO production the macrophage remains permissive for *L. pneumophila* replication. NO inhibits the growth of *L. pneumophila* in the lungs during the initial stages (within 3-5 days) of infection by limiting intrapulmonary replication prior to the development of *L. pneumophila* specific cellular immunity. Their results show that TLR9 is required for classical lung macrophage activation in intracellular bacterial infection and that TLR9 plays a critical role in the programming of the macrophage phenotype. They also demonstrated that host immunity could be restored in mutant mice by the adoptive transfer of syngeneic wild type DCs but this was not achieved using bone marrow derived DCs from TLR9$^{-/-}$ mice, which suggests that impaired DC function in TRL9$^{-/-}$ mice is responsible for the immune defects observed. In addition, they found that intra-

tracheal but not systemic treatment with CpG class A ODN was associated with a 7.5 fold reduction in *L. pneumophila* cfu counts at 72 hours after bacterial administration in A/J mice, and also describe similar results using the C-class CpG ODN 10101, suggesting that compartmental pre-treatment with a synthetic TLR9 agonist can stimulate protective innate immunity in murine *Legionella* pneumonia.

After establishing the presence of TLRs in primary cells of the respiratory system I went to look for the potential regulator called toll-interacting protein (tollip). Tollip m-RNA transcripts and protein were identified in the human colon carcinoma cell line T84 in accordance to the data published by Otte, J., and coworkers (2004) and Melmed, G., and colleagues, (2003). Following this, it was possible to identify tollip protein using confocal microscopy analysis in the human nasal epithelial cell line RPMI 2650. The cells stained strongly with tollip antibody not only in the cytoplasm, but also in the nucleolus. The morphological appearance of the stain in these cells is similar in distribution and localisation to that described by Li, T., et al, 2004, who showed that tollip localises to the cytoplasm of HeLa cells stably transfected with TLR4 and MD2 (HeLa-MAT). Further work after transfection with pEGFP-tollip in the same cells noted that tollip colocalises with pECFP-golgi, which suggests that tollip preferentially localies to the Golgi apparatus. Observations made by Brissoni, B., et al, (2006) in mouse embryonic fibroblasts (MEF) showed staining for tollip in the cytosol and suggested that this localisation is likely to be on late endosomes,. Moreover, Ciarrocchi, A., (2009) also demonstrated small aggregates of tollip in HeLa cells

in a perinuclear position probably corresponding to the Golgi apparatus or endosomes. They also demonstrated colocalisation between tollip and the protein SUMO-1 in human SAOS-2/IL-1R osteoblastoma cells (in which IL-1RI is constitutively expressed). Concluding that SUMO-1 and tollip are translocated into the nucleus and internalised into nuclear bodies. Furthermore, they found in the same type of cells that Daxx (a transcriptional co-repressor interacting with many sumoylated factors) interacts with tollip, given by their colocalization in defined nuclear structures suggesting a nuclear role as well as involvement in nuclear cytoplasmic trafficking.

During infection with *S. aureus* strain Newman, it was observed that RPMI 2650 cells constitutively express tollip. Although conventional PCR at 4 hours was not helpful in predicting changes in m-RNA transcription during infection, when a later time point such as 24 hours was assessed, it was observed that the bacteria had successfully overgrown and killed the cells. Exposure to ultraviolet light-killed bacteria was not associated with significant changes in *tollip* m-RNA transcription at both time points. In view of these results, quantitative PCR was performed in order to try to understand the effects of live infection by *S. aureus* on the nasal epithelial cells. The results confirmed that by 4 hours the infection not only affected *tollip* transcription but the whole cell integrity displayed by changes in *18S* and *GAPDH* during the active infective process. Infection of the nasal epithelial cell line RPMI 2650 with an MRSA strain also failed to show significant changes in *tollip* m-RNA transcription using RT-PCR.

In view of the multiple difficulties during the live prokaryotic-eukaryotic experiments, a sterile model was designed to assess the interaction with purified TLR ligands implicated in the infection process such as LPS, PGN and LTA. In these experiments it was noted that tollip protein levels in lysates from the RPMI 2650 cell line were very high and also remained unchanged after a 24 hour stimulation with bacterial products.

Tollip was also identified in the human type II alveolar epithelial cell line A549 using immunocytochemistry. The staining pattern was similar to that in RPMI 2650 cells: diffuse cytoplasmic with an associated nuclear staining. During live infection, conventional RT-PCR revealed a similar pattern in tollip transcripts: tollip was constitutively expressed and there were no significant changes after infection with two different doses of live bacteria. Intracellular tollip levels in the human type II alveolar epithelial cell line A549 were also very high but remained constant after 24 hour stimulation with bacterial ligands. Although this cell line appeared to be more responsive after stimulation, these changes were not statistically significant. Comparison of the tollip response after stimulation with bacterial products between the two cell lines did not show significant differences. A possible explanation for this finding is the fact that tollip's response to TLR ligands may happen in a relatively short period of time. The original description by Burns, K., in 2000 shows that tollip is rapidly associated and dissociated from its receptor complex. Tollip was detected two minutes after IL-1β stimulation but not at earlier or later time points in lysates from EL-4.6.10 cells. Furthermore,

Brissoni's work (2006), using MEF cells with wild type tollip and tollip$^{-/-}$ cells showed that 1 hour after IL-1β stimulation, the distribution of internalised IL-1RI was similar in both wild-type and tollip-deficient cells. However stimulation for longer periods (3-6 hours) showed that whilst in wild type cells IL-1RI was progressively more difficult to detect, in tollip-deficient cells significant levels of IL-1RI continued to accumulate in late endosomes. This accumulation could be reversed by stable reconstitution of tollip-deficient cells with tollip but not with empty vector, suggesting that tollip is required for efficient sorting of IL-1RI at late endosomes. Tollip's behaviour also appears to be related to the specific compartment in which it is found. The study by Otte, J., et al, 2004 used the intestinal epithelial cell lines T84, SW480 and Colo 205 and showed that after stimulation with LPS or LTA expression of tollip mRNA was up-regulated in both cell lines as early as 2 hours, reaching peak levels after 8 to 12 hours. This was also demonstrated at protein level. It is therefore likely that in my experiments, which involved a 24 hour incubation with the ligand, the cells achieved a balance in response to the ligands that stimulated them in the first instance faster, which is consistent with tollip's role as an endocytic adaptor. Sorting of the cargo to the early and late endosomal compartments happens in a relatively fast and highly coordinated manner in order to allow the cell to get back to its original homeostasis state and in doing so, achieving tolerance to the antigens expressed. One exception for this in my experiments was when the cells were incubated with peptidoglycan. Multiple deposits intracellularly appeared to stain positive with the tollip antibody possibly suggesting that peptidoglycan itself induces a change in

the conformation of tollip or affects the sorting of the cargo in the endosomal compartment. Future experiments could focus on addressing this issue in more detail.

Tollip protein was successfully identified in primary human nasal epithelial cells by immunocytochemistry. The pattern of the staining was punctate and distributed around the cytoplasm, probably in endosomes as previously described by Brissoni, B., et al, 2006 although markedly less strongly when compared to the cancerous cell line RPMI 2650. Nucleolar staining was less evident too. After stimulation with bacterial ligands, there was a change in the staining pattern, particularly with peptidoglycan from *S. aureus*, when the presence of multiple aggregates around the cytoplasm and in the nucleolus was observed; suggesting that stimulation with this ligand could affect the conformation of tollip or its ability to sort cargo in the endosomal compartment. Stimulation with TNF-α was not associated with any changes in the cellular staining pattern of tollip as expected.

Using real-time PCR it was possible to identify *tollip* m-RNA transcripts in primary nasal epithelial cells; although their response was different to that expected. LPS was not associated with an increase in mRNA transcription, and neither were PGN or LTA. These findings correlated with the immunocytochemistry findings in which there was not an increase in the protein expression of the protein.

Confocal microscopy analysis of primary bronchial epithelial cells showed similar findings to those from primary nasal cells, i.e. there was a diffuse punctate staining throughout the cytoplasm and nuclear staining was more subtle than in cancerous cell lines. These cells responded to peptidoglycan stimulation in a similar manner to the nasal epithelial cells, by forming tollip aggregates in the cytoplasm.

Immunocytochemistry in primary human type II alveolar epithelial cells also showed a diffuse cytoplasmic stain, but in contrast with the upper respiratory tract cells, the stain was stronger and distributed more in the perinuclear area of the cell. Nucleolar staining for tollip was more clearly defined. Due to technical difficulties obtaining and culturing these cells, it was not possible to fully assess the pattern of the tollip response to bacterial ligands, although this would be an area for further work in the future.

Taken together, the morphological appearances of tollip differs significantly when comparing cancer cell lines to primary cells in that the pattern of the staining is much stronger in cancer cells. This observation coincides with the observations made by Chen, Y., et al, 2007, in which, using a phosphoproteomics approach they validated tollip as a novel tyrosine kinase substrate implicated in breast cancer development. The relationship of this association is unknown, yet on the other hand, chronic inflammation and cancer usually go hand in hand and therefore it is likely that tollip might also have a role in cancer cell development.

Due to the technical difficulties in obtaining enough numbers of primary human epithelial cells, it was not possible to perform protein measurements on cell lysates in order to ascertain if there was a difference in the content of this protein intracellularly which could explain the difference in the staining pattern, although this is another aspect that could be pursued in the future.

Tollip's role in the lower respiratory tract seems to be similar to that described in the gastrointestinal system by Melmed, G., et al, 2003 and Otte, J., et al, 2004, as stimulation of primary human type II alveolar epithelial cells is associated with the up-regulation of tollip in response to LPS, whereas there appeared to be high variability among subjects after stimulation with PGN and LTA. Although these differences are not statistically significant, these could reflect biological variation among subjects.

Because of the nature of this study and of the primary cells used, it was not possible to ascertain the role that tollip plays in the interface with adaptive immunity, which certainly will be an area of further interest in the future.

When comparing the compartmental role that tollip could play in the upper and lower respiratory tract, it appeared that basal m-RNA transcripts were higher in primary nasal epithelial cells when compared to type II alveolar epithelial cells. In addition to this, stimulation with bacterial ligands was associated with a different response in the nasal to the alveolar compartment. In relation to LPS, nasal cells

appeared refractive to stimulation with TLR ligands. It could be that higher intracellular levels of tollip require an even higher dose of LPS to elicit a response. I noted that in the study by Otte, J., et al (2004), they used a dose of 2μg/ml whereas the LTA dose was the same (10μg/ml). These findings support the hypothesis that the nasal epithelium behaves as a compartment more tolerant of bacteria and it is highly likely that tollip plays an important role in allowing immunological tolerance at this site.

CHAPTER FIVE

RESULTS

5. Results

5.1. Role of the respiratory epithelium during *Staphylococcus aureus* colonisation

5.1.1. Introduction

Staphylococcus aureus is a bacterium that commonly colonises humans. The dynamics of colonisation have acquired increasing relevance recently since it has been observed that colonisation constitutes the determining step prior to infection by this organism (Dall'antonia, M., et al, 2005 and Watanabe, H., et al, 2000). The bacterium finds its niche in the anterior nares in the vestibulum nasi which consists of fully keratinised epithelium, apocrine sweat glands, sebaceous glands and hair follicles (Gluck, U., et al, 2000 and Wertheim, H., et al, 2005). It is accepted that approximately 20% of the population are persistent carriers, 30% are intermittent carriers and 50% are non carriers (Wertheim, H., et al, 2005).

Published literature about this subject varies, although the bacterial, epidemiological, genetic, and host factors in general have been studied extensively. However, the role of the epithelium in inducing an immunological response to the presence of this organism has been less well studied.

Not only is the physical presence of the whole bacterium required to elicit an immunological response. Assuming that under normal circumstances the innate immune system is able to contain the infection, some of the bacterial cells would be lysed and as a consequence will release bacterial DNA onto the epithelium.

Under these conditions, which I believe are physiological, the epithelium will not only recognize but mount a response to the presence of bacterial DNA.

The hypothesis that I would like to test are:

1. ***"the nasal epithelium plays an active role in the surveillance of microorganisms"***
2. "***Priming of the epithelium by bacterial DNA is associated with an inflammatory response".***

My aims are to look at the innate immune responses established by the nasal epithelium in association with carrier status and to try to establish if there are differences in how upper and lower respiratory tract epithelium reacts to the presence of bacterial DNA.

5.1.2. Definition and characterisation of nasal carriage of *S. aureus*

Nasal swabs were performed in all the subjects who consented to nasal brushings as described in the Materials and Methods section. Positive carrier status was defined by identification of colonies with a morphotype suggestive of *S. aureus* which were isolated in pure culture and identified using catalase, latex agglutination and tube coagulase tests as described in the Materials and Methods section (see Table 5.1 at the end of this chapter page 260).

A total of 47 nasal swabs were performed, and 3 subjects were sampled exclusively for flow cytometry. For detailed results on the identification of the bacterial strains associated with these experiments please refer to Tables 5.1 and 5.2 at the end of this chapter, pages 260 and 262 respectively.

5.1.3. Primary human nasal epithelial cells

After performing the nasal swabs in the subjects, I obtained primary human nasal epithelial cells using a nasal brushing technique as reported by McDougall, C., et al, 2008. Cells from healthy volunteers and patients who underwent partial lobectomy or pneumonectomy were obtained as described in the Materials and Methods section. Two major type of experiments were designed: the first one with the objective to establish the cellular type recovered from the subjects after performing nasal brushings and the second one, to establish a tissue culture model of primary human nasal epithelial cells in order to test their response to TLR ligands and to the presence of *S. aureus* in the nasal mucosa.

5.1.4. Flow cytometry of nasal brushings

Flow cytometric analysis was performed on cells recovered from nasal brushings performed on 20 healthy adult volunteers. A restricted panel of fluorescently labelled antibodies was used to ascertain cell phenotype. Nasal swabs were also performed and cultured to establish the colonisation status of each one of the subjects. This work is the result of collaboration with Dr. Paul Fitch from the

Schwarze/Immunity and Chronic Inflammation Group based at the QMRI, University of Edinburgh.

The antibody panel included antibodies raised against CD16 (FITC-labelled) for the identification of neutrophils, EpCAM (epithelial cell adhesion molecule; PE-labelled) to identify epithelial cells, CD14 (PERCP-labelled) for the identification of monocytes and macrophages and CD3 (APC-labelled) for the identification of lymphocytes (Figure 5.1). These markers are not exclusive to these cell types. CD16 can for example be found on a small subset of circulating monocytes and some natural killer cell subsets. Low level EpCAM expression can be present on some mucosal immune cell subsets and some highly activated cells can express both CD16 and CD14. However, given the limitation of small cell numbers in some samples and a 4 colour flow cytometer for analysis, this antibody panel was used. Where cell numbers permitted, staining was also performed for dendritic cells using antibodies raised against CD11c, CD123, MHC HLADR, LINEAGE (CD3, CD14, CD16, CD19, CD20, CD56), NK/B using anti CD16, CD20 and CD3 and for T lymphocyte subsets using antibodies raised against CD3, CD4, and CD8. However, the DC, NK/B and T lymphocyte stain results were not conclusive as insufficient cells were identified in either the colonized or uncolonized groups to enable any comparison to be made.

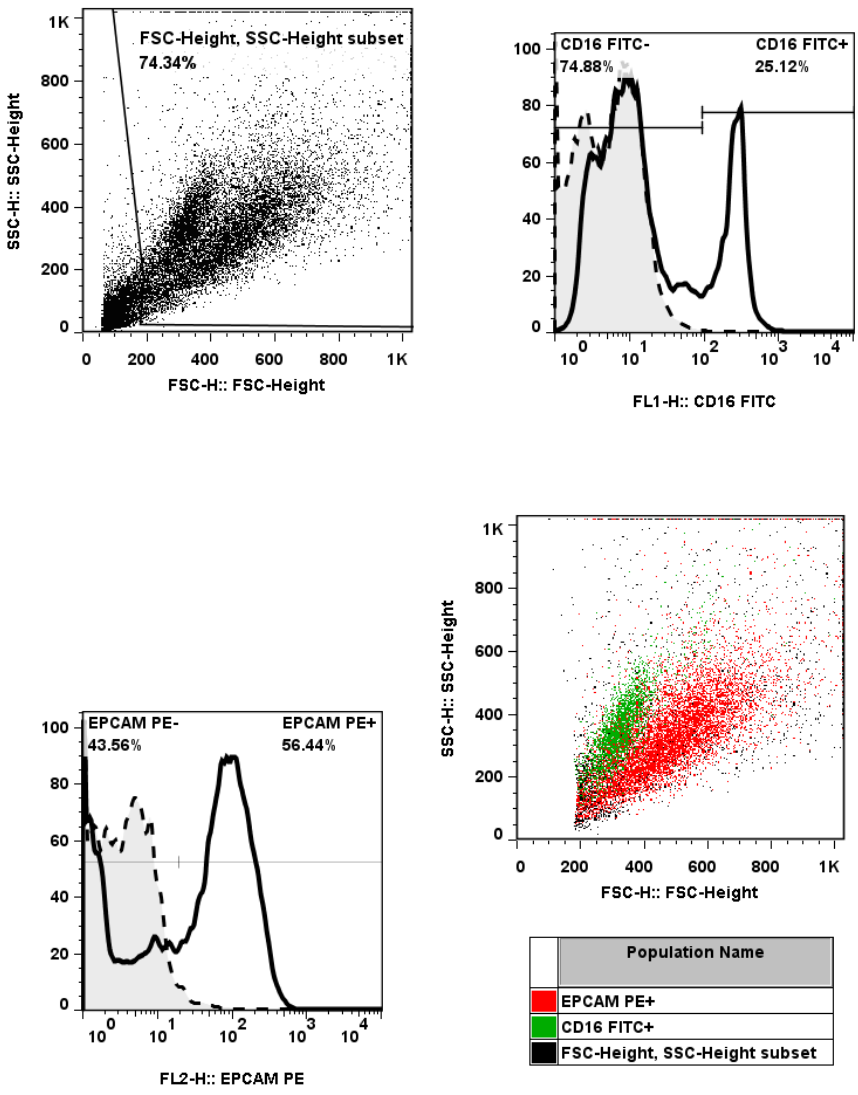


Figure 5.1 Flow cytometry analysis of a sample of primary nasal epithelial cells after brushing.

Cells were obtained after brushing of the inferior turbinate of the nose and resuspended in DMEM prior to antibody staining. Cells were washed in PBS-1%BSA containing 0.05% sodium azide and a single cell suspension obtained via ~15 passages through a 19g needle. Non-specific antibody binding was blocked with mouse serum. Antibodies were added (3µl per test with cells in 50-100µl volumes) for 30 minutes. Cells were then washed and fixed with 4% paraformaldehyde prior to analysis on a 4 colour FACS Calibur flow cytometer. The forward scatter (relative size) verses side scatter (relative granularity) gate above was defined using a heat- and UV-killed propidium iodide stained cell control to exclude dead cells. Cells at the bottom left are therefore debris and dead cells. Material in the top right hand corner represents clumped material.

After repated analysis, it was observed that the number of cells recovered from the first cohort of subjects had insufficient numbers of cells to make detailed conclusions. The latter group of volunteers (n=10) demonstrated that the dominant viable cell population recovered during the brushing was EpCAM positive cells, negative for CD14, CD16 and CD3 and therefore assumed to represent epithelium, median: 58.3% (range:24.7 – 74.6), followed by variable proportions of CD16 positive cells, assumed here to be neutrophils, median 5.6% (range: 0.8 – 32.7) and the number of CD14 positive cells assumed here to be macrophage/monocytes was small, as well as, a very small proportion of CD3 positive cells (lymphocytes) was identified in some volunteers. Results of the bacterial identification of the colonies can be seen in Table 5.2 at the end of this chapter.

In order to establish if there was a relationship in the amount of neutrophils recovered during brushing and the *S. aureus* colonisation status, the presence of CD16+ cells was analyzed. CD16+ cells are expressed as a percentage of total live cells recovered from the inferior turbinate of healthy volunteers. The data suggest that individuals colonized with *S. aureus* have a slightly higher proportion of neutrophils in their nasal mucosa in comparison to non-colonized individuals but this difference is not statistically significant (Figure 5.2).

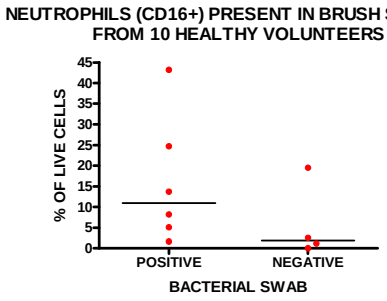


Figure 5.2 Association of *S. aureus* nasal carriage with presence of neutrophils during nasal brushing.
Cells were obtained after brushing of the inferior turbinate of the nose and resuspended in DMEM prior to antibody staining as described above and in the Materials and Methods section. *S. aureus* carrier status was performed as described in the Materials and Methods section. Bars are median values.

5.1.5. Description of healthy volunteers and patients for culture of primary nasal epithelial cells

A total of 44 subjects were consented for the isolation of primary nasal epithelial cells for culture. Of these 22 were healthy volunteers (HV) and 22 were patients undergoing partial lobectomy or pneumonectomy in the Cardiothoracic Surgical Department of the Royal Infirmary of Edinburgh. For further demographic information of the subjects involved please refer back to Chapter 3, section 3.2.2., page 103 and table 3.1 in page 105.

Overall, the sex and colonisation status were non statistically significant by Chi square ($p>0.05$) (Table 5.1). In the healthy volunteer group 10 subjects (45%) were colonised with *S. aureus*. For four subjects, this was the predominant morphotype found in culture. Twelve were non-colonised. In the patient group, only five patients were found colonised with *S. aureus* (22.7%) and 17 were non-colonised. The median number of morphologically different colonies in the colonised group with *S. aureus* was 3 (range 1-5), whereas in the group without *S. aureus* the equivalent median was also 3 (range 2-5). There were two subjects

in which an alpha- and a beta-haemolytic Streptococcus were found and three other subjects in which a Gram negative bacillus was isolated in culture. Since the main objective of this study was to assess the presence of *Staphylococcus aureus* in nasal swabs, no further attempts were made to make detailed identification of the other bacterial strains isolated. A predominant feature during the bacteriological identification process was the predominance of Micrococci species in the nasal swabs of individuals who did not have colonisation with *S. aureus*. Micrococci were found in 21 of the swabs from individuals without *S. aureus* colonisation, as compared with only 5 of the *S. aureus*-colonised individuals (p=0.0225) by Fisher's exact test. (Table 5.1)

5.2. Cytokine response to TLR ligands during nasal carriage with *S. aureus*

For the successful cultures, once the cells achieved confluence they were incubated with TLR ligands (LPS, *Staphylococcus aureus* PGN and LTA) and the pro-inflammatory cytokine TNF-α for 24 hours. Cell-free supernatants were taken and stored at -80^{o}C prior to assay, cells were lysed for RNA extraction as described in the Materials and Methods section.

For this part of the study, cells from 12 subjects were included: six subjects were found non colonised with *S. aureus* or any other pathogenic bacteria and six subjects were asymptomatic carriers for *S. aureus*. However, only one patient: P3

also had β-haemolytic streptococci spp isolated from a nasal swab) as seen in Table 5.4. The median age in the colonised group was 34 (range: 26 - 66), whereas in the non colonised group was 49.5 (range: 26 – 69). There were no statistical differences between the two groups for age (Mann Whitney test) or by sex or smoking status using Fisher's exact test. It would have been ideal to obtain higher numbers of subjects for this part of the study, although the numbers are small, the observations made are relevant for future studies.

Subject	*Age*	*Sex*	*Smoker*	*S. aureus colonisation*
HV1	42	M	N	N
HV2	31	M	N	**Y**
HV4	35	F	N	N
HV5	34	M	N	**Y**
HV8	26	F	N	**Y**
P3	66	M	Y	**Y**
HV10	33	M	N	**Y**
HV11	26	F	N	N
P6	57	M	Y	**Y**
P9	64	F	Y	N
P20	57	M	Y	N
P23	69	F	Y	N

Table 5.4 Demographic data for healthy volunteers and patients included in the colonisation study.
HV, Healthy volunteer; P, Patient.

5.2.1. IL-8

Median baseline IL-8 levels in non-colonised subjects were 274pg/ml (range: 192 – 647); whereas colonised subjects showed a significantly increased basal production of this cytokine with a median production of 1384pg/ml (range: 726 – 22456) (Figure 5.3).

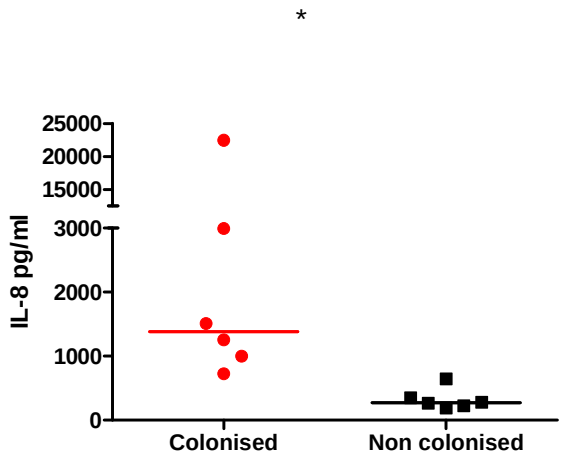


Figure 5.3 Constitutive IL-8 production by primary human nasal epithelial cells in relation to *S. aureus* nasal colonisation (n=12).
Cells were seeded in 6 well plates. After confluence cells were washed, and supernatants were removed after 24 hours. *S. aureus* colonisation was determined as discussed above.
Data are presented as individual data points and median (line).
Data were analysed using GraphPad Mann-whitney test.
Statistical significance (*) was established when p value <0.05

5.2.2. IL-6

Median constitutive IL-6 production was significantly increased during *S. aureus* carriage. IL-6 levels in non-colonised subjects were 16.2pg/ml (range: 5 – 28.6) whereas in colonised subjects it was 166pg/ml (range: 9.3 – 1982) (Figure 5.6).

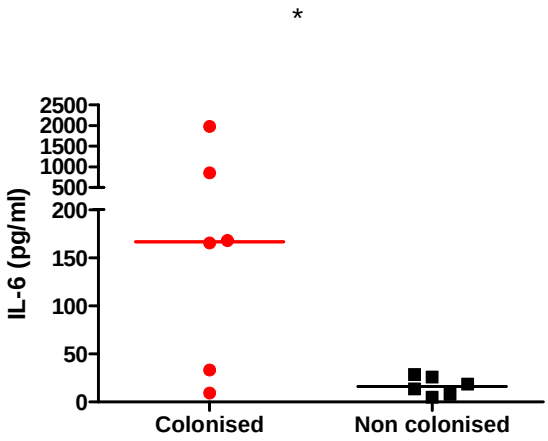


Figure 5.6 Constitutive IL-6 production by primary human nasal epithelial cells in relation to *S. aureus* nasal colonisation (n=12).
Cells were seeded in 6 well plates. After confluence, cells were washed and supernatants were removed after 24 hours. *S. aureus* colonisation was determined as discussed above.
Data are presented as individual data points and median (line).
Data were analysed using GraphPad Mann-whitney test.
Statistical significance (*) was established when p value <0.05

5.2.3. IL-1β

Median IL-1β production by non colonised subjects was 3pg/ml (range: 0 – 7.2), whilst subjects carrying S. aureus displayed significantly higher constitutive levels of IL-1β: median: 8.7pg/ml (range: 6.4 – 68.4). (Figure 5.9).

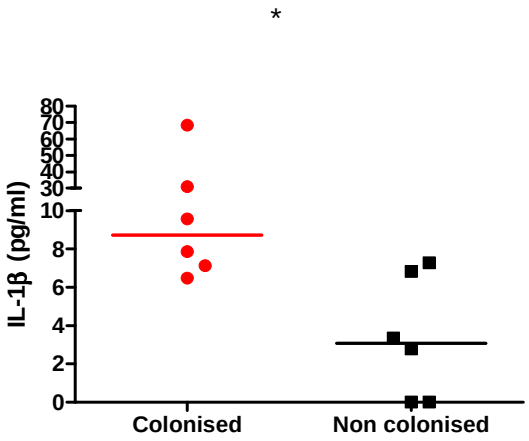


Figure 5.9 Constitutive IL-1β production by primary human nasal epithelial cells in relation to *S. aureus* nasal colonisation (n=12).
Cells were seeded in 6 well plates. After confluence, cells were washed and supernatants were removed after 24 hours. *S. aureus* colonisation was determined as discussed above.
Data are presented as individual data points and median (line).
Data were analysed using GraphPad Mann-Whitney test.
Statistical significance (*) was established when p value <0.05

The cells responded to stimulation with TLR ligands as well as with the pro-inflammatory cytokine TNF-α, reflecting the overall increase in the baseline levels of production associated with *S. aureus* carriage.

Median baseline levels of the cytokines IL-10, TNF-α and IL-12p70 were not affected by the colonisation status.

For a summary of the cytokine production in primary nasal epithelial cells in relation to *S. aureus* nasal carriage please see Table 5.5.

Cytokine	*S. aureus* carriage	Non Carrier	P value
	(pg/ml)	(pg/ml)	
IL-8	1384 (726 - 22456)	274 (192 - 647)	0.0022*
IL-6	166 (9.3 – 1982)	16.2 (5 – 28.6)	0.0260*
IL-1β	8.7 (6.4 – 68)	3.1 (0 – 7.2)	0.0152*
TNF-α	4.1 (1.7 – 46)	6.8 (0 – 10.9)	0.8182
IL-10	4.4 (3 – 16)	5.9 (2.6 – 18.7)	1
IL-12p70	5.5 (3.6 – 15.6)	8 (2.2 – 19.8)	0.93

Table 5.5 Cytokine production by primary nasal epithelial cells in association with *S. aureus* nasal carriage.
Data are presented are median cytokine concentration (range) for each cytokine studied
Statistical analysis was performed using Mann-Whitney test
Statistical significance (*) was defined when $p<0.05$.

5.3. Role of tollip during *S. aureus* colonisation

In order to address the question if tollip plays a role during *S. aureus* colonisation, primary nasal epithelial cells from healthy volunteers and patients were grown in culture. Bacterial identification in nasal swabs was performed, as previously described. Cells were left untreated or stimulated for 24 hours with TLR ligands such as LPS (positive control), PGN, LTA and the pro-inflammatory cytokine TNF-α (negative control). After completing incubation the cells were lysed and RNA extraction was performed. QPCR was performed for *tollip*, *18S* was used as the housekeeping gene, as described in the Materials and Methods section.

The median relative value for tollip m-RNA transcripts in non colonised subjects was 6.4 (range: 5 – 8.2), whereas in colonised subjects was 4.2 (range: 2.5 – 16.3). It was not possible to observe an association between tollip transcription and *S. aureus* carriage.

5.4. Cytokine response after stimulation of TLR9 in primary human respiratory epithelial cells

The effects of TLR9 stimulation in primary cells of the respiratory tract have not been extensively studied. We must start with the premise that an infection is a highly dynamic process during which complex interactions happen both at the host cellular level and at the pathogen level, while bacterial cells simultaneously multiply and die throughout. Once a bacterial cell dies its contents, including bacterial DNA, are presumably released into the surrounding lung parenchyma. Extracellular bacterial DNA may therefore play a role in the inflammatory response. Although there is evidence that bacterial DNA CpG motifs promote pro-inflammatory responses in the lower respiratory tract (Schwartz, D., et al, 1997), the specific response has not been evaluated in primary epithelial cells from the respiratory tract. The following experiments were designed to attempt to answer this question.

5.4.1. Cytokine response after stimulation of TLR9 in primary nasal epithelial cells

Human primary nasal epithelial cells were obtained from consented patients. For patient demographic details please see Table 5.6.

Subject	*Age*	*Sex*	*Smoker*	*Diagnosis*	*Culture Successful*
P10	71	M	Y	Squamous cell Ca	Y
P13	67	M	Y	Lung adenocarcinoma	Y
P15	61	M	N	Interstitial lung disease	Y
P16	60	F	N	Pleural fibroma	Y
P19	72	M	N	Interstitial lung disease	Y
Total					5

Table 5.6 Demographic details of patients from whom primary nasal epithelial cells were obtained for TLR9 stimulation experiments

Cells were extracted and cultured as described in the Materials and Methods section. After confluence was achieved, cells were washed and incubated for 24 hours with vehicle only (medium), CpG-C or the inhibitory oligonucleotide - ODN TTAGGG human TLR9 signaling inhibitor (InvivoGen, San Diego, California). The inhibitory ODN TTAGGG chosen belongs to the telomeric class

of inhibitory oligonucleotides, is a 25 mer, and its full sequence is as follows: 5'-TTT AGG GTT AGG GTT AGG GTT AGG G-3'.

The experiments were designed using two models: a non colonisation model and a colonisation model trying to mimic the physiological situation. In the non-colonised model cells received vehicle (culture media only) for 24 hours and were then challenged with CpG-C, LPS and LTA for a further 24 hours. In the model of colonisation, the cells were primed with CpG class C for 24 hours and then challenged for a further 24 hours with CpG-C, LPS, LTA or the inhibitory oligonucleotide (ODN). I was also keen to assess the effects that the inhibitory ODN could have on the cells and also included wells in which the cells were primed with ODN. After a total of 48 hours, cell-free supernatants were removed and stored at -80^{o}C until processed using the Becton Dickinson CBA Human Inflammation kit as per the manufacturer's instructions. Cell lysates were processed for total protein measurement using the Pierce assay as described in the Materials and Methods section. Cytokine concentration in supernatant was normalised to lysate protein content for each individual sample.

5.4.1.1. IL-8 and IL-6:

Median constitutive levels of IL-8 secretion in primary human nasal epithelial cells were 2984 pg/mg of protein (range: 362 - 26458 pg/mg of protein) and 1173 pg/mg of protein (range: 18.4 - 2761 pg/mg of protein) for IL-6.. These levels are approximately 10 and 40 fold higher respectively in comparison to the

24 hour time point experiments described in Chapter 4 section 4.2.5.1.2 page 173. This could be related to the longer time point as secreted cytokine accumulates in supernatant.

Priming with CpG-C was not associated with an increase in the secretion of IL-8 or IL-6 after further challenge of the cells with CpG-C, LPS or LTA. However, secretion of these cytokines appeared to have responded to down-regulation of TLR-9 as seen by a decrease in the secretion of these cytokines after stimulation with the inhibitor ODN.

5.4.1.2. IL-1β, IL-10, TNF-α and IL-12p70:

Median constitutive levels of IL-1β, IL-10, TNF-α and IL-12p70 appeared unchanged from the levels reported in the experiments at 24 hours described in Chapter 4, (section 4.2.5.1.2).

Priming with CpG-C at 24 hours was not an associated increase in the secretion of the above mentioned cytokines upon further challenge with CpG-C, LPS and LTA, although a decrease in the secretion of these cytokines was observed after challenge with ODN in some of the patient's cells.

5.4.2. Cytokine response after stimulation of TLR9 in primary type II alveolar epithelial cells :

Primary human type II alveolar epithelial cells were obtained from consented patients as described in the Materials and Methods section. Lung specimens were processed as described in the Materials and Methods section and single cells

were cultured onto type I collagen-coated plastic ware. All experiments were performed at passage 1. For a list of the subjects from whom primary cells were used please see Table 5.7.

Subject	*Age (Yr)*	*Sex*	*Lobe resected*	*Final diagnosis*	*Smoker*	*Cultures Successful*
8	63	F	L	Single lung metastasis from malignant Melanoma	N	Y
9	64	F	L	Squamous cell Ca	Y	Y
12	60	F	M	Non small cell lung Ca	Y	Y
14	77	F	RL	Lung Adenocarcinoma	N	Y

Table 5.7 Demographic details of patients from which primary type II alveolar epithelial cells were obtained for assessment of TLR9 function

After achieving confluence monolayers were washed and treated in the same way as previously described for primary nasal epithelial cells. Experiments were designed using the colonised and non-colonised models as explained in the previous section. Cells were stimulated with TLR ligands, and cell-free supernatants were removed and stored at -80^{o}C until analysed. Supernatants were processed using the Becton Dickinson CBA Human Inflammation kit as per the manufacturer's instructions. Monolayers were lysed and processed for total protein measurement using the Pierce method.

5.4.2.1. IL-8, IL-6 and IL-10:

Median constitutive levels of IL-8 in primary human type II alveolar epithelial cells were 14379 pg/mg of protein (range: 286 - 84309 pg/mg of protein) after 48 hours in culture. These levels are increased by approximately a 6 fold in comparison to the experiments performed at 24 hours in Chapter 4, section 4.2.5.2.2, page 176 during which, stimulation with CpG-C was associated with a significant increase in IL-8 secretion. Priming with CpG-C was associated with a slight increase in the secretion of IL-8 after challenge with CpG-C and LTA, whereas this was not observed after LPS challenge. Challenge with CpG-C+ODN induced a decrease in IL-8 secretion.

Median constitutive levels of IL-6 in primary human type II alveolar epithelial cells were 944 pg/mg of protein (range: 9.5 - 2715 pg/ mg of protein) at 48 hours. These levels are increased by approximately a 3 fold in comparison to the 24 hour experiments (Chapter 4, section 4.2.5.2.2). Priming with CpG-C was not associated with an increase the secretion of IL-6 after being challenged with CpG-C, LPS or LTA; inhibition of TLR9 by ODN was associated with a decrease in the secretion of IL-6.

Median baseline levels of IL-10 in primary human type II alveolar epithelial cells were 1088 pg/mg of protein (range: 9 – 1504 pg/mg of protein) at 48 hours. These levels appear greatly increased by approximately a 70 fold in comparison to the 24 hour experiments described previously (Chapter 4, section 4.2.5.2.2).

Stimulation with CpG-C was associated with an increase in the secretion of IL-10 into supernatant. Priming with CpG-C produced variable results after challenge of the cells with TLR ligands. Challenge with the TLR9 inhibitor ODN was associated with a decrease in the secretion of IL-10.

5.4.2.2. IL-1β, TNF-α and IL-12p70:

Median constitutive levels of IL-1β, TNF-α and IL-12p70 in primary type II alveolar epithelial cells remained equivalent from the 24 hour time point as described in Chapter 4, section 4.2.5.2.2. Priming with CpG-C was not associated with an increase in cytokine secretion. However, incubation with the TRL9 inhibitor ODN showed a slight decrease in the secretion of the above mentioned cytokines.

5.4.3. Comparison in the cytokine response between human primary nasal and lung epithelial cells to TLR9 stimulation and inhibition:

One of the main objectives of this research was to assess the differences in the response between cells from different compartments within the respiratory tract. In the following data, I will be comparing the cytokine production by cells from the upper respiratory tract (nasal) to cells from the lower respiratory tract (alveolar), when subjected to the same experimental conditions. The response

between unstimulated, TLR9-stimulated (CpG-C primed) cells and TLR9 inhibited (ODN TTAGGG primed) cells will be examined.

5.4.3.1. IL-8 and IL-6:

Median constitutive IL-8 production of this cytokine was higher in primary type II alveolar epithelial cells; which appeared to increase the secretion after incubation with CpG-C, although not in a statistically significant manner. Inhibition of TLR9 after incubation with the oligonucletide inhibitor ODN was associated with a decrease in IL-8 secretion.

Primary nasal epithelial cells appeared more refractive to stimulation with a TLR9 agonist. However, inhibition of TLR9 with ODN was associated with a decrease in IL-8 secretion.

IL-6 secretion displayed a similar pattern to the one described for IL-8 in both cell types.

5.4.3.2. IL-10:

Surprisingly, the type II primary human alveolar epithelial cells produced median constitutive levels of IL-10 that were higher in comparison to primary nasal epithelial cells. In response to stimulation of TLR9 the levels of IL-10 secreted by lung epithelial cells increased in comparison to primary nasal epithelial cells in which median levels of IL-10 secreted into supernatant remained unchanged. Inhibition of TLR9 after incubation with the ODN was associated with decrease in the secretion of this cytokine which appeared more marked in type II alveolar

cells in comparison to primary nasal epithelial cells in which IL-10 levels remained unchanged.

5.4.3.3. TNF-α, IL-1β and IL-12p70:

The constitutive production of TNF-α, IL-1β and IL-12p70 by human primary cells of the upper and lower respiratory tract was similarly low. Upon stimulation of these cells with a TLR9 agonist, there were modest increases in the production of TNF-α and IL-1β in both cell types. In contrast, TLR9 stimulation had no effect in IL-12p70 secretion.

Inhibition of TLR9 with the inhibitor ODN was not associated with changes in the production of any of the cytokines in mention.

For a summary of the cytokine responses from primary nasal and type II alveolar epithelial cells please refer to Table 5.8.

	Primary nasal epithelial cells			Primary type II alveolar epithelial cells		
	Vehicle (pg/mg protein)	CpG-C (pg/mg protein)	ODN (pg/mg protein)	Vehicle (pg/mg protein)	CpG-C (pg/mg protein)	ODN (pg/mg protein)
IL-8	2984 (362-26458)	2042 (1481-30313)	1279 (400-16347)	14379 (286-84309)	25364 (945 - 68854)	17713 (658 – 60261)
IL-6	1173 (18 – 2761)	930 (136 - 2010)	519 (22 – 1566)	944 (9.5 – 2715)	1271 (50 – 1704)	1070 (38 – 1790)
IL-1β	10 (0 – 390)	4.7 (3 – 427)	7.3 (0 – 356)	3 (1.7 -27)	7.7 (3.2 – 29)	5.7 (2.5 – 27)
TNF-α	1 (0.8 - 6.3)	1.2 (0 – 3.4)	0.9 (0 – 7.5)	2.5 (0- 4.7)	4.5 (2.4 – 5.9)	3.4 (1.2 – 7.7)
IL-10	1.4 (0 – 1559)	1.3 (0 – 930)	1.2 (1.2 – 3)	1088 (9.5 – 1504)	1252 (9.2 – 1969)	873.2 (6.3 – 1133)
IL-12p70	1.6 (1.1 – 6.6)	1.8 (1.7 – 3.5)	1.6 (0.7 – 7.8)	3 (1.8 – 5.2)	4.9 (4 – 7.6)	2.4 (0 -10.4)

Table 5.8 Summary of cytokine responses by primary nasal and type II alveolar epithelial cells to stimulation or inhibition of TLR9

5.5. Discussion:

I tried to make my project as translational as possible, therefore I spent the first two years designing the tools and optimising techniques and experiments with cell lines in order to try these in primary cells in an attempt to bring science as close to the bedside as possible. Nevertheless, despite a considerable effort, it became obvious that translation of science is a highly complex and inevitably slow process. As a result, some of the observations described in this chapter although novel, have the main limitation of not being a representative sample of the population since the numbers of subjects studied are rather small and definitive assumptions can not be made for some of the experiments. Nevertheless, these observations could constitute pilot data for follow up studies in the future.

Current medical practice has advanced greatly. As a result patients with diseases that used to be thought of like a “death sentence” now live longer. Parallel to these advances our internal bacterial reservoir has also adapted and evolved to the new challenges during this symbiotic relationship. As a result, new treatments introduced by medicine into man have also been followed by the adaptation of microorganisms to these new “environmental” conditions and we are now facing the threat of increasing antimicrobial resistance. One of the most relevant organisms involved in this transition is *Staphylococcus aureus*, which as discussed earlier has successfully developed antimicrobial resistance and

strains with the methicillin resistance gene (*mecA*) are now implicated in infections in the hospital as well as in the community setting (Woodford, N., and Livermore, D., 2009). The main reason why this bacterium represents a real threat to current clinical practice is because of its ability to colonise humans. The molecular mechanisms involved in colonisation are just starting to emerge. Some of the studies emphasise bacterial factors, but the specific role of the epithelium - particularly the nasal epithelium which is its main niche - has somehow been ignored.

In this study, I found 16 subjects (34%) colonised with *Staphylococcus aureus* in the anterior portion of the nose, in accordance with the current literature (Gordon, R., and Lowy, F., 2008; Van,Belkum, A., 2006 and Wertheim, H., et al, 2005). I did not establish the pattern of colonisation (permanent, transient or non-colonised), since that is out of the scope of this research. The bacterial identification process was performed identifying colony morphotypes plated in duplicate to avoid error. Predominant morphotype colonies were subcultured further and tested for Staphylococcal markers. *Staphylococcus aureus* identification was performed using two different methods in all the predominant morphotypes recovered from nasal swabs. While these methods were not exhaustive, they are in accordance with current standard operating procedures for the identification of *S. aureus* (HPA SOP, 2007). In the colonised group, there was one patient in which a beta-haemolytic Streptococcus species was isolated from a nasal swab.

It is important to mention that in 4 colonised subjects, *S. aureus* was the predominant bacterial morphotype isolated during bacteriological culture, whereas non-colonised subjects had more than two morphotypes isolated with predominance of *Micrococcus* species. This finding suggests that *Micrococcus* could have a protective role in the nasal epithelium by competing against *S. aureus* strains. Bacterial competition for the niche has been highlighted in the study by Dall'Antonia, M., et al, 2005, in which they showed that there is competition between methicillin-sensitive (MSSA) and methicillin-resistant *Staphylococcus aureus* (MRSA) strains in the anterior portion of the nose among a group of 680 patients in London. Furthermore, the role of bacterial interference in relation to *S. aureus* colonisation has also been studied when Shinefield, H., et al, 1963 observed that inoculation with a non-pathogenic strain of *S. aureus* (strain 502A) protected neonates from colonisation with a pathogenic *S. aureus* strain (phage type 80/81). In addition, other bacteria such as *Lactobacillus* spp and *Corynebacterium* spp have proven to successfully interfere with *S. aureus* colonisation in an animal model as well as in human carriers (Gan, B., et al, 2002 and Uehara,Y., et al, 2000).

Primary human nasal epithelial cells were obtained by gentle brushing of the medial aspect of the inferior turbinate. In order to ascertain the phenotype of the cells extracted, flow cytometry was performed demonstrating that the dominant

population (median: 75%) were EpCAM positive cells, negative for CD14, CD16 and CD3, and highly likely to represent epithelium, followed by CD16 positive cells likely to be neutrophils, and CD14 positive cells likely to be monocytes/macrophages. A very small proportion of CD3 positive cells assumed to be lymphocytes were identified in some volunteers. It is important to note that these markers are not strictly exclusive of the cell types mentioned since CD16 can be found is subsets of monocytes and natural killer cells, and mucosal immune cell subsets can express EpCAM at a low level. This data agrees with the data published by Ramanathan, M., and Lane, A., (2007) who compared nasal brushings with ethmoid mucosa specimens for the isolation of primary nasal epithelial cells and showed by flow cytometry that brush biopsy specimens contained 75% epithelial cells.

When we tried to establish a relationship between the different cell populations recovered from nasal brushing specimens and nasal colonisation with *S. aureus* an attempt was made to determine whether colonised subjects had an increased neutrophil count in nasal brushings. The results indicate that colonised individuals have a slightly higher proportion of neutrophils in their nasal mucosa in comparison to non-colonised subjects, although the difference was not statistically significant, probably due to the small size of the sample studied. The study by Cole, A., et al, 1999 showed that concentrations of neutrophil-derived host defence peptides were significantly elevated in nasal fluid from carriers of *S. aureus* in their nasal mucosa, suggesting that neutrophil recruitment happens in

response to nasal colonisation. They also mention that light microscopy examination of the nasal fluid with Wright's stain confirmed that neutrophils were present in carrier and acute rhinitis donor fluids but not in fluid from normal donors.

I then went on to assess the cytokine response to TLR ligands during nasal carriage of *S. aureus*. It appears that the presence of this bacterium in vivo "primes" the cells *in vitro* (and for a considerable amount of time after isolation) toward a pro-inflammatory phenotype. Constitutive levels of IL-8, IL-6 and IL-1β were significantly higher in association with nasal carriage. This response was enhanced further after stimulation with ligands predominantly from *S. aureus* such as peptidoglycan. Stimulation with the pro-inflammatory cytokine TNF-α was also associated with a rise in secretion of IL-8, IL-6 and IL-1β. It was also noted that constitutive levels of these cytokines were higher in healthy volunteers in comparison to patients' cells probably due to volunteers being younger and non-smokers. Smoking itself has been associated with alterations in TLRs expression in respiratory epithelial cells, which could account for the differences in the cytokine responses observed between non smokers and smoker subjects (MacRedmond, R., et al, 2007). Their cells appeared to respond in a more efficient manner in comparison to patients' cells upon stimulation with PGN and TNF-α. The high constitutive production of primarily pro-inflammatory cytokines such as IL-8, IL-6 and IL-1β in supernatants from subjects colonised with *S. aureus* reveals that although colonisation appears to behave in an

asymptomatic manner, at epithelial level there is increased inflammation even after a relatively long period of time ex-vivo in the cells. Data published by Ramanathan, M., and Lane, A., 2007, comparing the expression of immune markers in primary nasal epithelial cells studied acutely or after six weeks in culture showed that primary epithelial cells maintain their innate immune receptor expression profile and that alterations in innate immune gene expression may be intrinsic to the individual set of epithelial cells. Although my study only shows the response of epithelial cells sampled once, all experiments were performed at passage 2 and median time to reach confluence prior to performing an experiment was 3 weeks. Therefore, although the cells were grown ex-vivo, they were used in experiments in conditions as close as possible to their original precursors in order to avoid confounding factors due to multiple passages. The study by O'Brien, G., et al (2006) compared primary nasal epithelial cultures in asthmatic patients to healthy donors, and found that asthmatic patients produced significantly higher IL-8 secretion levels in cell culture supernatants. Priming of the cells with the pro-inflammatory cytokine IFN-γ was associated with a further increase in the secretion of IL-8. In addition, they also exposed the cells to *S. aureus* enterotoxins A an B at non-toxic concentrations and observed an associated increase in the production of IL-8, while they found no differences in TNF-α, RANTES and eotaxin production. Asthma is considered an inflammatory disease of the airways caused by hypersensitivity mediated by IgE, associated with an increase in local cytokines and infiltration of the epithelium by eosinophils, basophils and T_H2 cells. It is likely that the pro-inflammatory

environment associated with the disease itself had greatly influenced the responses observed ex-vivo.

Viral infections have been associated with exaggerated IL-8 and IL-6 responses in airway epithelium-like NCI-H292 cells. Roger, T., et al, 2004, they observed that parainfluenza virus type 4a (PIV-4a) enhances production of IL-8 and IL-6 in airway cells by increasing the DNA-binding activity for the transcription factors NF-κB and CCAAT-enhancer binding protein (C/EBP). This appears to be the cause of the first peak in the cytokine secretion, whereas the second peak appears to be related to a reduction in IL-8 and IL-6 degradation. These factors have been considered to play an important role in the virus-induced potentiation of inflammation in the airway epithelium. Furthermore, in the study by Lopez-Souza, N., et al, 2004, using primary nasal epithelial cultures, an increase in the output of IL-8 and IL-6 following rhinovirus infection was observed. This was also associated with an increase in mRNA levels for these cytokines, which correlated with levels of viral mRNA.

Further evidence for epithelium acting as a surveillance organ in establishing an adaptive immunological response was provided by Ritz, H., and colleagues (1984) when they found an association with high serum titres of antibodies to Staphylocococcal toxic shock antigen (TSA) in subjects colonised with a TSA-producing strain of *S. aureus*. More recently, Verkaik, N., et al, 2010, have published a study in which they followed 57 children from birth to 2 years of

age. They found that IgG and IgA levels against *S. aureus* proteins were significantly higher among children colonised with *S. aureus*.

Taken together, these findings suggest that nasal colonisation with *S. aureus*, far from being a silent process, is the result of successful innate as well as adaptive immune responses in order to contain the infection by this pathogen. Furthermore, some evidence suggests that the development of antibodies against *S. aureus* strains has a protective role (Wertheim, H., et al, 2004).

It was also noted that the cells did not respond to stimulation with LPS or LTA, this is probably due to the physiological down-regulation of the specific receptors involved in their recognition as described previously in Chapter 3.

There were no major changes in the basal production of TNF-α, IL-10 or IL-12p70, although patients' cells displayed slightly higher constitutive levels of these cytokines in supernatant in comparison to healthy volunteers' cells. The very low levels of IL-12p70 are probably associated with the purity of the cultures since this cytokine is predominantly produced by macrophages and dendritic cells.

It is important also to highlight that the results were obtained using monolayers submerged in culture, which are associated with poor differentiation. Although my study is in accordance with the current published literature, ideally it would

be desirable to have grown the cells at an air-liquid culture interface and although it was not possible for this study, it could be achieved in the future.

The negative regulator of the TLR signalling pathway tollip did not appear to have a significant role in relation to nasal colonisation with *Staphylococcus aureus*..

The presence of TLR9 was demonstrated earlier during this work in primary cells of the upper as well as the lower respiratory tract (sections 4.2.5.1 page 171 and 4.2.5.2, page 174 respectively). The first report in the literature of the effects of bacterial DNA in the lower respiratory tract was from Krieg's group in 1997, three years prior to the discovery of TLR9 by Akira's group (Hemmi, H., et al, 2000). They found that bacterial DNA is pro-inflammatory when compared to eukaryotic DNA and these effects are independent of endotoxin contamination. Furthermore, the effects were significantly reduced by DNA methylation. They recovered DNA from the sputum of patients with cystic fibrosis and after intratracheal instillation of 50μg of this DNA in the lower respiratory tract of mice were able to reproduce acute pulmonary inflammation given by the increase in the concentration of total cells and neutrophils in lavage fluid. A previous report also by Krieg's group showed that mice pre-treated with bacterial DNA had much higher mortality when challenged with LPS probably due to an IFN-γ mediated mechanism (Cowdery, J., et al, 1996).

The study by Platz, J., et al, 2004, found that TLR9 is expressed in airway epithelium. TLR9 mRNA levels were relatively low in comparison to those in the human B cell lymphoma cell line Raji. They observed up-regulation of the pro-inflammatory cytokines IL-6 and IL-8 which appeared to be related to an increase in NF-κB dependent activity.

The role of TLR9 during the development of bacterial pneumonia has been studied by Albiger, B., et al (2007) and Bhan, U., and colleagues in 2008. Both studies conclude that TLR9 is essential for the successful clearance of bacteria within the lungs. The latter study suggested that compartmental pre-treatment with a synthetic TLR agonist can stimulate protective innate immunity during infection with the Gram negative *Legionella pneumophila*.

Since CpG oligonucleotides are currently being considered as potential vaccine adjuvant candidates, and having shown previously that their main action appears to be increasing the secretion of IL-8 predominantly in primary type II alveolar epithelial cells (whilst secretion of IL-6 and IL-10 remained unchanged), I wanted to explore how upper and lower respiratory tract cells would respond when TLR9 activators or inhibitors were used either as priming agents (colonisation experiments) or administered simultaneously with CpG-ODN.

High constitutive secretion of IL-8 and IL-6 by primary nasal epithelial cells was observed during the colonisation experiments. This could reflect accumulation of

these cytokines in cultured supernatant since the time course of the experiments was doubled in comparison to the experiments shown in section 4.2.5.1.2 (page 173).

My experiments suggest that the nasal epithelial response to TLR9 activation is likely to be dependent on other local inflammatory factors and that in an attempt to control an undesirable inflammatory response locally, the nasal epithelium could behave in an anergic manner. Nevertheless, the incubation with the TLR9 inhibitor ODN TTAGGG was associated with a decrease in the secretion of IL-8 and IL-6 in cultured supernatant.

During the quest for the answer to the immune effects of DNA dichotomy (i.e. bacterial DNA being stimulatory and mammalian being inhibitory) Pisetsky's group noted that synthetic oligonucleotides that contain poly-G sequences could block bacterial DNA-induced activation (Halpern, M., and Pisetsky, D., 1995). Furthermore, the inhibitory sequences were over-expressed in certain strains of adenovirus (Krieg, A., et al, 1998). Further work established that the CpG motif itself is not required for inhibition, but that three consecutive G nucleotides are necessary for inhibition and that the length of the oligonucletide itself is one of the main determining factors contributing to the activity. Currently, inhibitory ODNs are classified as class B-broadly active, R-restricted activity or G-telomeric. For a detailed explanation of the characteristics of each class please refer to Lenert, P., 2010.

The inhibitory oligonucleotide that I chose to use in my study belongs to the telomeric class. It is known that the ends of linear eukaryotic chromosomes have specialised DNA protein structures called telomeres. These telomeres contain large numbers of single-stranded hexanucleotide repeats of the sequence TTAGGG, which physiologically protect mammalian chromosomes from degradation (Gursel, I., et al, 2003; Blasco, M., et al, 1999). Moreover, whilst telomeric-rich G repeats are present at high frequency in the genomes of eukaryotes, they are extremely rare in bacteria and it is likely that during evolution this mechanism was selected as a manner to differentiate host versus foreign DNA. Furthermore, the presence of these sequences could be available in high local concentrations in tissues following the death of host cells and this could be a mechanism for limiting immune activation after cellular death in tissues. The study led by Gursel, demonstrates that repetitive TTAGGG motifs down-regulate the response to CpG DNA by blocking the co-localisation of CpG DNA with TLR9 in endosomal vesicles. TTAGGG multimers inhibited all forms of CpG-induced immune activation including the stimulation of B lymphocytes, dendritic cells and macrophages. Mammalian telomeres are not the only sequence motifs with suppressive properties. Although they claim that they are non toxic, they also say that they did not inhibit mitogen- or LPS-induced immune activation. They did not prevent the binding or uptake of CpG DNA by immune cells. They interfered with the maturation of endosomal vesicles and the colocalisation of CpG DNA with TLR9 in the vesicles. Other studies have shown that very early events in CpG-mediated signalling (NF-κB translocation) are

inhibited by suppressive ODN (Yamada, H., et al, 2002; Lenert, P., et al, 2001). In vivo, suppressive ODN blocked CpG-induced cytokine production by more than 75% (Gursel, I., et al, 2003).

When studying type II alveolar epithelial cells in the colonisation experiments it was also possible to observe high constitutive levels of IL-8, IL-6 and IL-10. Constitutive secretion of these cytokines has been reported previously and is probably related to the role that the alveolar epithelium plays in surveillance against foreign and potentially dangerous particles/microorganisms. The high levels in these experiments could also reflect an accumulation of these cytokines in cultured supernatant as a result of the longer time of incubation that the experiments took (48 hours) in comparison when the experiments performed at 24 hours as seen in section 4.2.5.2.2 (page 176). The response of these cells to TLR9 stimulation was quite different; CpG-C stimulation of these cells was followed by an increase in the median secretion predominantly of IL-8, although IL-6 and IL-10 also displayed an increase in their median secretion. Furthermore, incubation with the inhibitor ODN was associated with a decrease of the secretion of these cytokines.

The study by El Kebir, D., et al, 2009, looked at the activation of HUVEC cells by bacterial DNA and observed it to be a potent amplifier of the inflammatory response. In addition, they also observed that unmethylated CpG motifs evoked endothelial cell activation associated with increased nuclear translocation of NF-

κB and subsequent up-regulation of adhesion molecules such as ICAM-1 and E-selectin. Their study coincides with mine in that they also used a telomeric ODN with the same sequence used in my study (TTAGGG) which as described before has the ability to diffuse into cells and disrupt the co-localisation of CpG DNA with TLR9 in endosomal vesicles, therefore affecting cellular uptake. They observed a significant decrease in the production of IL-8 after priming with the inhibitory ODN followed by challenge with CpG DNA. They also found that the inhibitory ODN produced a higher degree of inhibition of TLR9-mediated NK-κB activation in HUVECs than the endosomal acidification inhibitors used.

The study published by Trieu, A., et al, 2009 demonstrated that inhibitory ODN 2114 amplifies the intracellular *Salmonella typhimurium* load in mouse bone marrow-derived macrophages in a TLR9-independent manner. They also found that although its activity did not interfered with bacterial LPS (TLR4) or zymosan (TLR2/6/Dectin-1) responses, it partially suppressed the response to the bacterial lipoprotein analogue Pam_3Cys (TLR1/2), as well as to the TLR7 agonist R837. Since TLR2 is required for the response to *S. typhimurium*, they conclude that it is likely that inhibitory ODN interferes with the host antimicrobial response by suppressing TLR2. A report by Stacey, K., et al, 2003, also demonstrated that ODN 2114 potently inhibited CpG-mediated activation of a NK-κB reporter in RAW264 murine macrophages.

These reports coincide with my findings in that, ODN TTAGGG incubation was associated with decreased secretion of IL-8 and IL-6 in primary nasal epithelial cells as well as a trend toward decrease predominantly in the secretion of IL-8, IL-6 and IL-10 in primary type II alveolar epithelial cells. These findings were consistent whether cells were only primed with ODN or if they were also challenged with CpG-C simultaneously; further studies need to look further into this observation. Whilst most studies published to date have produced evidence for the role of this inhibitory ODN in cells of the innate immune system (macrophages, dendritic cells and lymphocytes), this report highlights the potential deleterious effects that TLR9 inhibition could have on the host immune's response if TLR9 is inhibited at epithelial level since it could predispose the host towards an increasing risk of invasive Gram positive bacterial infections if these molecules were to be administered locally in the respiratory epithelium.

Although the differences in the responses of both types of epithelial cells appear genuine, they lack statistical significance probably due to the low numbers of patients recruited for this part of the study

Subject	Colonies	Catalase	Staphytect plus	Tube coagulase	Colonisation status	Micrococci
1	3	pos/pos/pos	neg/neg/neg	neg/neg/neg	Negative	yes
2	3	pos/pos/pos	neg/neg/pos	neg/neg/pos	Positive high	
3	3	pos/pos/pos	neg/neg/neg	neg/neg/neg	Negative	yes
4	3	pos/pos/pos	neg/neg/neg	neg/neg/neg	Negative	yes
5	4	pos/pos/pos/pos	neg/pos/pos/neg	neg/pos/pos/pos	Positive-high	
6 (P1)	3	pos/pos/pos	neg/neg/neg	neg/neg/neg	Negative	yes
7 (P2)	2	pos/pos	neg/neg	neg/neg	Negative	
8	3	pos/pos/pos	pos/pos/neg	pos/pos/neg	Positive - high	
9 (P3)	5	neg/pos/pos/pos/pos	n/a/neg/neg/pos/neg	n/a/neg/neg/pos/neg	Positive	yes
10	2	pos/pos	pos/neg	pos/neg	Positive - high	
11	2	pos/pos	neg/neg	neg/neg	Negative	
12	3	pos/pos/pos	neg/neg/neg	neg/neg/neg	Negative	yes
13 (P4)	3	pos/pos/pos	neg/neg/neg	neg/neg/neg	Negative	yes
14	3	pos/pos/pos	neg/neg/neg	neg/neg/neg	Negative	yes
15	1	pos	pos	pos	Positive - high	
16	4	gnb/pos/pos/pos	n-a/pos/neg/pos	n-a/pos/neg/pos	Positive	yes
17	3	pos/pos/pos	neg/neg/neg	neg/neg/neg	Negative	yes
18	3	pos/pos/pos	neg/neg/neg	neg/neg/neg	Negative	yes
19	4	pos/pos/pos/pos	neg/neg/neg/neg	neg/neg/neg/neg	Negative	yes
20 (P5)	3	pos/pos/pos	pos/neg/neg	pos/neg/neg	Positive	
21 (P6)	3	pos/pos/pos	pos/neg/neg	pos/neg/neg	Positive	
22	3	pos/pos/pos	neg/neg/neg	neg/neg/neg	Negative	yes
23	4	pos/pos/pos/pos	pos/pos/neg/neg	pos/pos/neg/neg	Positive	yes
24 (P7)	3	pos/pos/pos	neg/neg/neg	neg/neg/neg	Negative	
25 (P8)	4	pos/pos/pos/pos	neg/neg/neg/neg	neg/neg/neg/neg	Negative	yes
26(P9)	4	pos/pos/pos/pos	neg/neg/neg/neg	neg/neg/neg/neg	Negative	
27	4	pos/pos/pos/pos	neg/neg/neg/neg	neg/neg/neg/neg	Negative	yes
28	2	pos/pos	pos/neg	pos/neg	Positive - high	

29 (P10)	3	pos/pos/pos	neg/neg/neg	neg/neg/neg	Negative	yes
30	2	pos/pos	pos/pos	pos/pos	Positive	
31	5	gnb/pos/pos/pos/pos	n-a/neg/neg/neg/neg	n-a/neg/neg/neg/neg	Negative	yes
32	4	pos/pos/pos/pos	pos/neg/neg/neg	pos/neg/neg/neg	Positive	yes
33	4	pos/pos/pos/pos	neg/neg/neg/neg	neg/neg/neg/neg	Negative	
34	4	pos/pos/pos/pos	neg/neg/neg/neg	neg/neg/neg/neg	Negative	
35	1	pos	pos	pos	Positive - high	
36 (P11)	3	pos/pos/pos	neg/neg/neg	neg/neg/neg	Negative	
37(P12)	3	pos/pos/pos	neg/neg/equivocal	neg/neg/neg	Negative	
38(P13)	3	pos/pos/pos	neg/neg/neg	neg/neg/neg	Negative	yes
39(P14)	N/A					
40(P15)	3	pos/pos/pos	pos/neg/neg	pos/neg/neg	Positive	yes
41(P16)	3	pos/pos/pos	neg/neg/equivocal	neg/neg/neg	Negative	yes
42(P17)	4	pos/pos/pos/pos	neg/neg/neg/neg	neg/neg/neg/neg	Negative	yes
43(P18)	3	pos/pos/pos	neg/neg/neg	neg/neg/neg	Negative	
44(P19)	5	neg/pos/pos/pos/pos	n-a/neg/neg/neg/neg	n-a/neg/neg/neg/neg	Negative	yes
45(P 20)	4	pos/pos/pos/pos	neg/neg/neg/neg	neg/neg/neg/neg	Negative	yes
46(P 21)	4	gnb/pos/pos/pos	n-a/neg/neg/neg	n-a/neg/neg/neg	Negative	yes
47(P 22)	3	pos/pos/pos	pos/pos/pos/neg	pos/pos/pos/neg	Positive	
48(P 23)	3	pos/pos/pos	neg/neg/neg	neg/neg/neg	Negative	

Table 5.1 Results of bacterial identification in nasal swabs from healthy volunteers and patients.
Gnb, Gram-negative bacilli; n-a, non applicable; pos, positive result; neg, negative result.

Volunteer	Plate #	Colony types	Catalase	Staphytect plus	Tube coagulase 4 h	Tube coagulase 24 h	Colonisation status
HV 2	1	Large white colonies	Positive	Negative	Negative	Negative	
		Large grey colonies	Positive	Positive	Positive	Positive	Positive
	2	Large white colonies	Positive	Positive	Positive	Positive	
		Smaller grey colonies	Positive	Negative	Negative	Negative	
HV 3	1	Small white colonies	Positive	Positive?? Equiv	Negative	Negative	Negative
		Small grey colonies	Positive	Positive	Negative	Negative	
		Micrococci	Positive	Negative	Negative	Negative	
	2	Large white colonies	Positive	Negative	Negative	Negative	
		Small grey colonies	Positive	Positive?? Equiv	Negative	Negative	
		Small white colonies	Positive	Negative	Negative	Negative	
		Micrococci	Positive	Negative	Negative	Negative	
HV 4	1	Large grey colonies	Positive	Negative	Negative	Negative	Negative
		Large white colonies	Positive	Negative	Negative	Negative	
		Micrococci	Positive	Negative	Negative	Negative	
	2	Small white colonies	Positive	Negative	Negative	Negative	
		Large grey colonies	Positive	Negative	Negative	Negative	
		One yellow colony	Positive	Negative	Negative	Negative	
		Micrococci	Positive	Negative	Negative	Negative	
HV 5	1	Large yellow colonies	Positive	Positive	Positive	Positive	Positive
		Small white colonies	Positive	Negative	Negative	Negative	
		Large grey colonies	Positive	Negative	Negative	Negative	
	2	Large yellow colonies	Positive	Positive	Positive	Positive	
		Small white colonies	Positive	Negative	Negative	Negative	
		Large grey colonies	Positive	Negative	Negative	Negative	
HV 8	1	Large yellow colonies	Positive	Positive	Positive	Positive	Positive
		Small white colonies	Positive	Negative	Negative	Negative	
		Small grey colonies	Positive	Negative	Negative	Negative	
		Micrococci	Positive	Negative	Negative	Negative	
	2	Contaminated					
HV 10	1	Large yellow colonies	Positive	Positive	Positive	Positive	Positive

	2	Large yellow colonies	Positive	Positive	Positive	Positive	
		White colonies	Positive	Negative	Negative	Negative	
HV 12	1	Small grey colonies	Positive	Positive?? Equiv	Negative	Negative	Negative
		Yellow colonies	Positive	Negative	Negative	Negative	
		Micrococci	Positive	Negative	Negative	Negative	
	2	Grey colonies	Positive	Negative	Negative	Negative	
		Small white colonies	Positive	Negative	Negative	Negative	
		Micrococci	Positive	Negative	Negative	Negative	
HV 14	1	Large white colonies	Positive	Negative	Negative	Negative	Negative
		Large grey colonies	Positive	Negative	Negative	Negative	
		Micrococci	Positive	Negative	Negative	Negative	
	2	Large white colonies	Positive	Negative	Negative	Negative	
		Grey colonies	Positive	Negative	Negative	Negative	
		Micrococci	Positive	Negative	Negative	Negative	
HV 15	1	Large yellow colonies	Positive	Positive	Positive	Positive	Positive
	2	Large yellow colonies	Positive	Positive	Positive	Positive	
HV 16	1	Large cream ? S. aureus	Positive	Positive	Positive	Positive	Positive
		Large green colony ?gnb		N/A	N/A	N/A	
		Middle size white colony	Positive	Negative	Negative	Negative	
		Micrococci	Positive	Negative	Negative	Negative	
	2	Large yellow colonies	Positive	Positive	Positive	Positive	
		White colonies	Positive	Negative	Negative	Negative	
		Micrococci	Positive	Negative	Negative	Negative	
HV 17	1	Large grey colonies	Positive	Negative	Negative	Negative	Negative
		Small white colonies	Positive	Negative	Negative	Negative	
		Micrococci	Positive	Negative	Negative	Negative	
	2	Micrococci	Positive	Negative	Negative	Negative	
		Grey colonies	Positive	Negative	Negative	Negative	
		Large white colonies	Positive	Negative	Negative	Negative	
HV 18	1	White colonies	Positive	Negative	Negative	Negative	Negative
		Micrococci	Positive	Negative	Negative	Negative	
		Grey colonies	Positive	Negative	Negative	Negative	
	2	Micrococci	Positive	Negative	Negative	Negative	

		White colonies	Positive	Negative	Negative	Negative	
		Grey colonies	Positive	Negative	Negative	Negative	
HV 22	1	Small grey colonies	Positive	Negative	Negative	Negative	Negative
		White colonies	Positive	Negative	Negative	Negative	
		Micrococci	Positive	Negative	Negative	Negative	
	2	Grey colonies	Positive	Negative	Negative	Negative	
		White colonies	Positive	Negative	Negative	Negative	
		Micrococci	Positive	Negative	Negative	Negative	
HV 28	1	Yellow colony ?S.aureus	Positive	Positive	Positive	Positive	Positive
		White colonies	Positive	Negative	Negative	Negative	
	2	Large yellow colony	Positive	Positive	Positive	Positive	
		Large white colonies	Positive	Negative	Negative	Negative	
HV 30	1	Large yellow colonies ?S.aureus	Positive	Positive	Positive	Positive	Positive
		Small grey colonies	Positive	Equivocal	Negative	Negative	
	2	Large yellow colonies ?S.aureus	Positive	Positive	Positive	Positive	
		White colonies	Positive	Negative	Negative	Negative	
HV 31	1	Large mucoid colonies gnb		N/A	N/A	N/A	
		Grey colonies	Positive	Negative	Negative	Negative	Negative
		Yellow colonies	Positive	Negative	Negative	Negative	
	2	Mucoid colony ?gnb	Positive	Negative	Negative	Negative	
		Yellow colonies	Positive	Negative	Negative	Negative	
		White colonies	Positive	Negative	Negative	Negative	
		Micrococci	Positive	Negative	Negative	Negative	
HV 32	1	Large yellow colony ?S.aureus	Positive	Positive	Positive	Positive	Positive
		White colonies	Positive	Negative	Negative	Negative	
		Alpha-haemolytic Strep	Negative	N/A	N/A	N/A	
	2	Large yellow colony ?S.aureus	Positive	Positive	Positive	Positive	
		White colonies	Positive	Positive	Positive	Positive	
		Micrococci	Positive	Negative	Negative	Negative	
HV 33	1	Yellow colony ?S.aureus	Positive	Negative	Negative	Negative	Negative
		Large white colonies	Positive	Negative	Negative	Negative	
		Large grey colonies	Positive	Negative	Negative	Negative	
		Micrococci	Positive	Negative	Negative	Negative	

	2	Large grey colonies	Positive	Negative	Negative	Negative	
		Large white colonies	Positive	Negative	Negative	Negative	
		Micrococci	Positive	Negative	Negative	Negative	
HV 34	1	Large yellow colonies ?S.aureus	Positive	Negative	Negative	Negative	Negative
		Small grey colonies	Positive	Negative	Negative	Negative	
		Small white colonies	Positive	Negative	Negative	Negative	
		Micrococci	Positive	Negative	Negative	Negative	
	2	Large yellow colonies ?S.aureus	Positive	Negative	Negative	Negative	
		Grey colonies	Positive	Negative	Negative	Negative	
		Small white colonies	Positive	Negative	Negative	Negative	
		Micrococci	Positive	Negative	Negative	Negative	
HV 35	1	Single dominant yellow col ?S.aureus	Positive	Positive	Positive	Positive	Positive
	2	Single dominant yellow col ?S.aureus	Positive	Positive	Positive	Positive	

Table 5.2 Results of the identification of bacterial colonisation in nasal swabs from healthy volunteers for flow cytometr

Reference List

Abbas A, Lichtman A. Cytokines. In: Saunders, editor. Cellular and Molecular Immunology. Elsevier, 2003: 243-274.

Aderem A, Ulevitch RJ. Toll-like receptors in the induction of the innate immune response. Nature 2000; 406(6797):782-787.

Agnese DM, Calvano JE, Hahm SJ et al. Human toll-like receptor 4 mutations but not CD14 polymorphisms are associated with an increased risk of gram-negative infections. J Infect Dis 2002; 186(10):1522-1525.

Akira S, Takeda K. Toll-like receptor signalling. Nat Rev Immunol 2004; 4(7):499-511.

Albiger B, Dahlberg S, Sandgren A et al. Toll-like receptor 9 acts at an early stage in host defence against pneumococcal infection. Cell Microbiol 2007; 9(3):633-644.

Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature 2001; 413(6857):732-738.

Aly R, Maibach HI, Shinefield HR, Mandel AD. Staphylococcus aureus carriage in twins. Am J Dis Child 1974; 127(4):486-488.

Ammerlaan H, Seifert H, Harbarth S et al. Adequacy of antimicrobial treatment and outcome of Staphylococcus aureus bacteremia in 9 Western European countries. Clin Infect Dis 2009; 49(7):997-1005.

Andersson SG, Karlberg O, Canback B, Kurland CG. On the origin of mitochondria: a genomics perspective. Philos Trans R Soc Lond B Biol Sci 2003; 358(1429):165-177.

Applied Biosystems. Guide to performing relative quantitation of gene expression using real-time quatitative PCR. 1-70. 2008.
Ref Type: Generic

Araya MT, Siah A, Mateo D et al. Selection and evaluation of housekeeping genes for haemocytes of soft-shell clams (Mya arenaria) challenged with Vibrio splendidus. J Invertebr Pathol 2008; 99(3):326-331.

Armstrong-Esther CA. Carriage patterns of Staphylococcus aureus in a healthy non-hospital population of adults and children. Ann Hum Biol 1976; 3(3):221-227.

Armstrong L, Medford AR, Uppington KM et al. Expression of functional toll-like receptor-2 and -4 on alveolar epithelial cells. Am J Respir Cell Mol Biol 2004; 31(2):241-245.

Artero A, Zaragoza R, Camarena JJ, Sancho S, Gonzalez R, Nogueira JM. Prognostic factors of mortality in patients with community-acquired bloodstream infection with severe sepsis and septic shock. J Crit Care 2010.

Baba T, Bae T, Schneewind O, Takeuchi F, Hiramatsu K. Genome sequence of Staphylococcus aureus strain Newman and comparative analysis of staphylococcal genomes: polymorphism and evolution of two major pathogenicity islands. J Bacteriol 2008; 190(1):300-310.

Bai S, Yang T, Abbruscato TJ, Ahsan F. Evaluation of human nasal RPMI 2650 cells grown at an air-liquid interface as a model for nasal drug transport studies. J Pharm Sci 2008; 97(3):1165-1178.

Balzar S, Chu HW, Silkoff P et al. Increased TGF-beta2 in severe asthma with eosinophilia. J Allergy Clin Immunol 2005; 115(1):110-117.

Bantel H, Sinha B, Domschke W, Peters G, Schulze-Osthoff K, Janicke RU. alpha-Toxin is a mediator of Staphylococcus aureus-induced cell death and activates caspases via the intrinsic death pathway independently of death receptor signaling. J Cell Biol 2001; 155(4):637-648.

Barber M, Hayhoe FG, ., Whitehead JE. Penicillin-resistant staphylococcal infection in a maternity hospital. Lancet 1949; 2(6590):1120-1125.

Barton GM, Kagan JC. A cell biological view of Toll-like receptor function: regulation through compartmentalization. Nat Rev Immunol 2009; 9(8):535-542.

Bauer S, Kirschning CJ, Hacker H et al. Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. Proc Natl Acad Sci U S A 2001; 98(16):9237-9242.

Bayles KW, Wesson CA, Liou LE, Fox LK, Bohach GA, Trumble WR. Intracellular Staphylococcus aureus escapes the endosome and induces apoptosis in epithelial cells. Infect Immun 1998; 66(1):336-342.

Bekeredjian-Ding I, Roth SI, Gilles S et al. T cell-independent, TLR-induced IL-12p70 production in primary human monocytes. J Immunol 2006; 176(12):7438-7446.

Berube J, Bourdon C, Yao Y, Rousseau S. Distinct intracellular signaling pathways control the synthesis of IL-8 and RANTES in TLR1/TLR2, TLR3 or NOD1 activated human airway epithelial cells. Cell Signal 2009; 21(3):448-456.

Beveridge TJ. Ultrastructure, chemistry, and function of the bacterial wall. Int Rev Cytol 1981; 72:229-317.

Bhan U, Trujillo G, Lyn-Kew K et al. Toll-like receptor 9 regulates the lung macrophage phenotype and host immunity in murine pneumonia caused by Legionella pneumophila. Infect Immun 2008; 76(7):2895-2904.

Blasco MA, Gasser SM, Lingner J. Telomeres and telomerase. Genes Dev 1999; 13(18):2353-2359.

Boone DL, Turer EE, Lee EG et al. The ubiquitin-modifying enzyme A20 is required for termination of Toll-like receptor responses. Nat Immunol 2004; 5(10):1052-1060.

Boucher RC, Yankaskas JR, Cotton CU, Knowles MR, Stutts MJ. Cell culture approaches to the investigation of human airway ion transport. Eur J Respir Dis Suppl 1987; 153:59-67.

Boyce JM, Havill NL, Maria B. Frequency and possible infection control implications of gastrointestinal colonization with methicillin-resistant Staphylococcus aureus. J Clin Microbiol 2005; 43(12):5992-5995.

Brissoni B, Agostini L, Kropf M et al. Intracellular trafficking of interleukin-1 receptor I requires Tollip. Curr Biol 2006; 16(22):2265-2270.

Bryant CE, Spring DR, Gangloff M, Gay NJ. The molecular basis of the host response to lipopolysaccharide. Nat Rev Microbiol 2010; 8(1):8-14.

Bulut Y, Faure E, Thomas L, Equils O, Arditi M. Cooperation of Toll-like receptor 2 and 6 for cellular activation by soluble tuberculosis factor and Borrelia burgdorferi outer surface protein A lipoprotein: role of Toll-interacting protein and IL-1 receptor signaling molecules in Toll-like receptor 2 signaling. J Immunol 2001; 167(2):987-994.

Burns K, Martinon F, Esslinger C et al. MyD88, an adapter protein involved in interleukin-1 signaling. J Biol Chem 1998; 273(20):12203-12209.

Burns K, Clatworthy J, Martin L et al. Tollip, a new component of the IL-1RI pathway, links IRAK to the IL-1 receptor. Nat Cell Biol 2000; 2(6):346-351.

Burns K, Janssens S, Brissoni B, Olivos N, Beyaert R, Tschopp J. Inhibition of interleukin 1 receptor/Toll-like receptor signaling through the alternatively spliced, short form of MyD88 is due to its failure to recruit IRAK-4. J Exp Med 2003; 197(2):263-268.

Burvall K, Palmberg L, Larsson K. Effects by 8-bromo-cyclicAMP on basal and organic dust-induced release of interleukin-6 and interleukin-8 in A549 human airway epithelial cells. Respir Med 2003; 97(1):46-50.

Carey BM, Dooley M, Weedle R, Clynes M. Production of autostimulatory growth factors by the human carcinoma line, RPMI 2650. In Vitro Cell Dev Biol 1993; 29A(2):153-160.

Cario E, Podolsky DK. Intestinal epithelial TOLLerance versus inTOLLerance of commensals. Mol Immunol 2005; 42(8):887-893.

Carpenter S, O'Neill LA. How important are Toll-like receptors for antimicrobial responses? Cell Microbiol 2007; 9(8):1891-1901.

Castiglia CL, Flint SJ. Effects of adenovirus infection on rRNA synthesis and maturation in HeLa cells. Mol Cell Biol 1983; 3(4):662-671.

Cazac BB, Roes J. TGF-beta receptor controls B cell responsiveness and induction of IgA in vivo. Immunity 2000; 13(4):443-451.

Chambers HF. The changing epidemiology of Staphylococcus aureus? Emerg Infect Dis 2001; 7(2):178-182.

Chambers HF. Community-associated MRSA--resistance and virulence converge. N Engl J Med 2005; 352(14):1485-1487.

Chambers HF, DeLeo FR. Waves of resistance: Staphylococcus aureus in the antibiotic era. Nat Rev Microbiol 2009; 7(9):629-641.

Chang L, Karin M. Mammalian MAP kinase signalling cascades. Nature 2001; 410(6824):37-40.

Chen C, Lee WH, Zhong L, Liu CP. Regulatory T cells can mediate their function through the stimulation of APCs to produce immunosuppressive nitric oxide. J Immunol 2006; 176(6):3449-3460.

Chen Y, Choong LY, Lin Q et al. Differential expression of novel tyrosine kinase substrates during breast cancer development. Mol Cell Proteomics 2007; 6(12):2072-2087.

Cheon IS, Woo SS, Kang SS et al. Peptidoglycan-mediated IL-8 expression in human alveolar type II epithelial cells requires lipid raft formation and MAPK activation. Mol Immunol 2008; 45(6):1665-1673.

Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987; 162(1):156-159.

Christensen SR, Kashgarian M, Alexopoulou L, Flavell RA, Akira S, Shlomchik MJ. Toll-like receptor 9 controls anti-DNA autoantibody production in murine lupus. J Exp Med 2005; 202(2):321-331.

Christensen SR, Shupe J, Nickerson K, Kashgarian M, Flavell RA, Shlomchik MJ. Toll-like receptor 7 and TLR9 dictate autoantibody specificity and have opposing

inflammatory and regulatory roles in a murine model of lupus. Immunity 2006; 25(3):417-428.

Ciarrocchi A, D'Angelo R, Cordiglieri C et al. Tollip is a mediator of protein sumoylation. PLoS One 2009; 4(2):e4404.

Cole AM, Dewan P, Ganz T. Innate antimicrobial activity of nasal secretions. Infect Immun 1999; 67(7):3267-3275.

Cole AM, Tahk S, Oren A et al. Determinants of Staphylococcus aureus nasal carriage. Clin Diagn Lab Immunol 2001; 8(6):1064-1069.

Cowdery JS, Chace JH, Yi AK, Krieg AM. Bacterial DNA induces NK cells to produce IFN-gamma in vivo and increases the toxicity of lipopolysaccharides. J Immunol 1996; 156(12):4570-4575.

Crapo JD, Barry BE, Gehr P, Bachofen M, Weibel ER. Cell number and cell characteristics of the normal human lung. Am Rev Respir Dis 1982; 126(2):332-337.

Crompton G, Haslett C, Chilvers E. Diseases of the respiratory system. In: Haslett C, Chilvers E, Hunter J, Boon N, editors. Davidson's Principles and practice of Medicine. Churchill Livingstone, 1999: 303-392.

da Silva MC, Zahm JM, Gras D et al. Dynamic interaction between airway epithelial cells and Staphylococcus aureus. Am J Physiol Lung Cell Mol Physiol 2004; 287(3):L543-L551.

Dall'antonia M, Coen PG, Wilks M, Whiley A, Millar M. Competition between methicillin-sensitive and -resistant Staphylococcus aureus in the anterior nares. J Hosp Infect 2005; 61(1):62-67.

Danforth JM, Strieter RM, Kunkel SL, Arenberg DA, VanOtteren GM, Standiford TJ. Macrophage inflammatory protein-1 alpha expression in vivo and in vitro: the role of lipoteichoic acid. Clin Immunol Immunopathol 1995; 74(1):77-83.

Dang NH, Torimoto Y, Sugita K et al. Cell surface modulation of CD26 by anti-1F7 monoclonal antibody. Analysis of surface expression and human T cell activation. J Immunol 1990; 145(12):3963-3971.

Dang NH, Torimoto Y, Shimamura K et al. 1F7 (CD26): a marker of thymic maturation involved in the differential regulation of the CD3 and CD2 pathways of human thymocyte activation. J Immunol 1991; 147(9):2825-2832.

Datta R, Huang SS. Risk of infection and death due to methicillin-resistant Staphylococcus aureus in long-term carriers. Clin Infect Dis 2008; 47(2):176-181.

De FA, Brun R, Felix H, Vonderscher J, Rummelt A. Evaluation of the human cell line RPMI 2650 as an in vitro nasal model. Rhinology 1995; 33(4):194-198.

DeRyke CA, Lodise TP, Jr., Rybak MJ, McKinnon PS. Epidemiology, treatment, and outcomes of nosocomial bacteremic Staphylococcus aureus pneumonia. Chest 2005; 128(3):1414-1422.

Dharmsathaphorn K, McRoberts JA, Mandel KG, Tisdale LD, Masui H. A human colonic tumor cell line that maintains vectorial electrolyte transport. Am J Physiol 1984; 246(2 Pt 1):G204-G208.

Dheda K, Huggett JF, Bustin SA, Johnson MA, Rook G, Zumla A. Validation of housekeeping genes for normalizing RNA expression in real-time PCR. Biotechniques 2004; 37(1):112-119.

dib-Conquy M, Adrie C, Fitting C, Gattolliat O, Beyaert R, Cavaillon JM. Up-regulation of MyD88s and SIGIRR, molecules inhibiting Toll-like receptor signaling, in monocytes from septic patients. Crit Care Med 2006; 34(9):2377-2385.

Didierlaurent A, Brissoni B, Velin D et al. Tollip regulates proinflammatory responses to interleukin-1 and lipopolysaccharide. Mol Cell Biol 2006; 26(3):735-742.

Diederen BM, Kluytmans JA. The emergence of infections with community-associated methicillin resistant Staphylococcus aureus. J Infect 2006; 52(3):157-168.

Dobbs LG. Isolation and culture of alveolar type II cells. Am J Physiol 1990; 258(4 Pt 1):L134-L147.

Donahue JA, Baldwin JN. Hemolysin and leukocidin production by 80/81 strains of Staphylococcus aureus. J Infect Dis 1966; 116(3):324-328.

Drage MG, Pecora ND, Hise AG et al. TLR2 and its co-receptors determine responses of macrophages and dendritic cells to lipoproteins of Mycobacterium tuberculosis. Cell Immunol 2009; 258(1):29-37.

Draing C, Sigel S, Deininger S et al. Cytokine induction by Gram-positive bacteria. Immunobiology 2008; 213(3-4):285-296.

Droemann D, Goldmann T, Branscheid D et al. Toll-like receptor 2 is expressed by alveolar epithelial cells type II and macrophages in the human lung. Histochem Cell Biol 2003; 119(2):103-108.

Dubuquoy L, Jansson EA, Deeb S et al. Impaired expression of peroxisome proliferator-activated receptor gamma in ulcerative colitis. Gastroenterology 2003; 124(5):1265-1276.

Dubuquoy L, Rousseaux C, Thuru X et al. PPARgamma as a new therapeutic target in inflammatory bowel diseases. Gut 2006; 55(9):1341-1349.

Duthie E, Lorenz L. Staphylococcal coagulase; mode of action and antigenicity. J Gen Microbiol 1952; 6(1-2):95-107.

Edelson JD, Shannon JM, Mason RJ. Alkaline phosphatase: a marker of alveolar type II cell differentiation. Am Rev Respir Dis 1988; 138(5):1268-1275.

Ehrhardt RO, Strober W, Harriman GR. Effect of transforming growth factor (TGF)-beta 1 on IgA isotype expression. TGF-beta 1 induces a small increase in sIgA+ B cells regardless of the method of B cell activation. J Immunol 1992; 148(12):3830-3836.

Eisenberg E, Levanon EY. Human housekeeping genes are compact. Trends Genet 2003; 19(7):362-365.

El KD, Jozsef L, Pan W, Wang L, Filep JG. Bacterial DNA activates endothelial cells and promotes neutrophil adherence through TLR9 signaling. J Immunol 2009; 182(7):4386-4394.

Enright MC, Robinson DA, Randle G, Feil EJ, Grundmann H, Spratt BG. The evolutionary history of methicillin-resistant Staphylococcus aureus (MRSA). Proc Natl Acad Sci U S A 2002; 99(11):7687-7692.

Fong CY, Pang L, Holland E, Knox AJ. TGF-beta1 stimulates IL-8 release, COX-2 expression, and PGE(2) release in human airway smooth muscle cells. Am J Physiol Lung Cell Mol Physiol 2000; 279(1):L201-L207.

Foster KA, Oster CG, Mayer MM, Avery ML, Audus KL. Characterization of the A549 cell line as a type II pulmonary epithelial cell model for drug metabolism. Exp Cell Res 1998; 243(2):359-366.

Fuchs S, Hollins AJ, Laue M et al. Differentiation of human alveolar epithelial cells in primary culture: morphological characterization and synthesis of caveolin-1 and surfactant protein-C. Cell Tissue Res 2003; 311(1):31-45.

Gan BS, Kim J, Reid G, Cadieux P, Howard JC. Lactobacillus fermentum RC-14 inhibits Staphylococcus aureus infection of surgical implants in rats. J Infect Dis 2002; 185(9):1369-1372.

Gay NJ, Keith FJ. Drosophila Toll and IL-1 receptor. Nature 1991; 351(6325):355-356.

Gentry M, Taormina J, Pyles RB et al. Role of primary human alveolar epithelial cells in host defense against Francisella tularensis infection. Infect Immun 2007; 75(8):3969-3978.

Gilchrist M, Thorsson V, Li B et al. Systems biology approaches identify ATF3 as a negative regulator of Toll-like receptor 4. Nature 2006; 441(7090):173-178.

Gioannini TL, Teghanemt A, Zhang D et al. Isolation of an endotoxin-MD-2 complex that produces Toll-like receptor 4-dependent cell activation at picomolar concentrations. Proc Natl Acad Sci U S A 2004; 101(12):4186-4191.

Girardin SE, Boneca IG, Viala J et al. Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. J Biol Chem 2003; 278(11):8869-8872.

Gluck U, Gebbers JO. The nose as bacterial reservoir: important differences between the vestibule and cavity. Laryngoscope 2000; 110(3 Pt 1):426-428.

Goffin C, Ghuysen JM. Biochemistry and comparative genomics of SxxK superfamily acyltransferases offer a clue to the mycobacterial paradox: presence of penicillin-susceptible target proteins versus lack of efficiency of penicillin as therapeutic agent. Microbiol Mol Biol Rev 2002; 66(4):702-38, table.

Gonzalez-Zorn B, Senna JP, Fiette L et al. Bacterial and host factors implicated in nasal carriage of methicillin-resistant Staphylococcus aureus in mice. Infect Immun 2005; 73(3):1847-1851.

Gordon RJ, Lowy FD. Pathogenesis of methicillin-resistant Staphylococcus aureus infection. Clin Infect Dis 2008; 46 Suppl 5:S350-S359.

Gorelik L, Flavell RA. Abrogation of TGFbeta signaling in T cells leads to spontaneous T cell differentiation and autoimmune disease. Immunity 2000; 12(2):171-181.

Greene CM, Carroll TP, Smith SG et al. TLR-induced inflammation in cystic fibrosis and non-cystic fibrosis airway epithelial cells. J Immunol 2005; 174(3):1638-1646.

Gruber A, Mancek M, Wagner H, Kirschning CJ, Jerala R. Structural model of MD-2 and functional role of its basic amino acid clusters involved in cellular lipopolysaccharide recognition. J Biol Chem 2004; 279(27):28475-28482.

Guha M, Mackman N. LPS induction of gene expression in human monocytes. Cell Signal 2001; 13(2):85-94.

Guillot L, Medjane S, Le-Barillec K et al. Response of human pulmonary epithelial cells to lipopolysaccharide involves Toll-like receptor 4 (TLR4)-dependent signaling pathways: evidence for an intracellular compartmentalization of TLR4. J Biol Chem 2004; 279(4):2712-2718.

Gursel I, Gursel M, Yamada H, Ishii KJ, Takeshita F, Klinman DM. Repetitive elements in mammalian telomeres suppress bacterial DNA-induced immune activation. J Immunol 2003; 171(3):1393-1400.

Guss B, Leander K, Hellman U, Uhlen M, Sjoquist J, Lindberg M. Analysis of protein A encoded by a mutated gene of Staphylococcus aureus Cowan I. Eur J Biochem 1985; 153(3):579-585.

Hacker H, Vabulas RM, Takeuchi O, Hoshino K, Akira S, Wagner H. Immune cell activation by bacterial CpG-DNA through myeloid differentiation marker 88 and tumor necrosis factor receptor-associated factor (TRAF)6. J Exp Med 2000; 192(4):595-600.

Halpern MD, Pisetsky DS. In vitro inhibition of murine IFN gamma production by phosphorothioate deoxyguanosine oligomers. Immunopharmacology 1995; 29(1):47-52.

Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000; 100(1):57-70.

Hashimoto C, Hudson KL, Anderson KV. The Toll gene of Drosophila, required for dorsal-ventral embryonic polarity, appears to encode a transmembrane protein. Cell 1988; 52(2):269-279.

Hay RT. SUMO: a history of modification. Mol Cell 2005; 18(1):1-12.

Hayashi F, Means TK, Luster AD. Toll-like receptors stimulate human neutrophil function. Blood 2003; 102(7):2660-2669.

Hayden MK, Rezai K, Hayes RA, Lolans K, Quinn JP, Weinstein RA. Development of Daptomycin resistance in vivo in methicillin-resistant Staphylococcus aureus. J Clin Microbiol 2005; 43(10):5285-5287.

Hemmi H, Takeuchi O, Kawai T et al. A Toll-like receptor recognizes bacterial DNA. Nature 2000; 408(6813):740-745.

Hemmi H, Kaisho T, Takeuchi O et al. Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. Nat Immunol 2002; 3(2):196-200.

Hessle CC, Andersson B, Wold AE. Gram-positive and Gram-negative bacteria elicit different patterns of pro-inflammatory cytokines in human monocytes. Cytokine 2005; 30(6):311-318.

Heumann D, Barras C, Severin A, Glauser MP, Tomasz A. Gram-positive cell walls stimulate synthesis of tumor necrosis factor alpha and interleukin-6 by human monocytes. Infect Immun 1994; 62(7):2715-2721.

Hidron AI, Edwards JR, Patel J et al. NHSN annual update: antimicrobial-resistant pathogens associated with healthcare-associated infections: annual summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2006-2007. Infect Control Hosp Epidemiol 2008; 29(11):996-1011.

Hiemstra PS, Maassen RJ, Stolk J, Heinzel-Wieland R, Steffens GJ, Dijkman JH. Antibacterial activity of antileukoprotease. Infect Immun 1996; 64(11):4520-4524.

Hiramatsu K, Hanaki H, Ino T, Yabuta K, Oguri T, Tenover FC. Methicillin-resistant Staphylococcus aureus clinical strain with reduced vancomycin susceptibility. J Antimicrob Chemother 1997; 40(1):135-136.

Hoeksma A, Winkler K. The normal flora of the nose in twins. Acta Leiden 1963; 32:123-133.

Holden MT, Feil EJ, Lindsay JA et al. Complete genomes of two clinical Staphylococcus aureus strains: evidence for the rapid evolution of virulence and drug resistance. Proc Natl Acad Sci U S A 2004; 101(26):9786-9791.

Holmes CL, Russell JA, Walley KR. Genetic polymorphisms in sepsis and septic shock: role in prognosis and potential for therapy. Chest 2003; 124(3):1103-1115.

Holt PG, Sedgwick JD. Suppression of IgE responses following antigen inhalation: a natural homeostatic mechanism which limits sensitization to aeroallergens. Immunol Today 1987; 8:14-15.

Holt PG, Strickland DH, Wikstrom ME, Jahnsen FL. Regulation of immunological homeostasis in the respiratory tract. Nat Rev Immunol 2008; 8(2):142-152.

Holt R, Stewart G. Techniques for the rapid and sensitive detection of penicillinase. J Clin Pathol 1963; 16:263-267.

Hornef MW, Frisan T, Vandewalle A, Normark S, Richter-Dahlfors A. Toll-like receptor 4 resides in the Golgi apparatus and colocalizes with internalized lipopolysaccharide in intestinal epithelial cells. J Exp Med 2002; 195(5):559-570.

Horng T, Barton GM, Flavell RA, Medzhitov R. The adaptor molecule TIRAP provides signalling specificity for Toll-like receptors. Nature 2002; 420(6913):329-333.

Hornung V, Rothenfusser S, Britsch S et al. Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. J Immunol 2002; 168(9):4531-4537.

Hu Y, Li T, Wang Y et al. Tollip attenuated the hypertrophic response of cardiomyocytes induced by IL-1beta. Front Biosci 2009; 14:2747-2756.

Huang SS, Hinrichsen VL, Stulgis Lea. Methicillin-resistant Staphylococcus aureus infection in the year following detection of carriage. Program and abstracts of the society of Healthcare Epidemiology of America Annual Meeting (Chicago) [Abstract 157]. 2006.
Ref Type: Abstract

Huang SS, Platt R. Risk of methicillin-resistant Staphylococcus aureus infection after previous infection or colonization. Clin Infect Dis 2003; 36(3):281-285.

Hussain T, Nasreen N, Lai Y, Bellew BF, Antony VB, Mohammed KA. Innate immune responses in murine pleural mesothelial cells: Toll-like receptor-2 dependent induction of beta-defensin-2 by staphylococcal peptidoglycan. Am J Physiol Lung Cell Mol Physiol 2008; 295(3):L461-L470.

Imler JL, Hoffmann JA. Signaling mechanisms in the antimicrobial host defense of Drosophila. Curr Opin Microbiol 2000; 3(1):16-22.

Iwaki D, Mitsuzawa H, Murakami S et al. The extracellular toll-like receptor 2 domain directly binds peptidoglycan derived from Staphylococcus aureus. J Biol Chem 2002; 277(27):24315-24320.

Iwami KI, Matsuguchi T, Masuda A, Kikuchi T, Musikacharoen T, Yoshikai Y. Cutting edge: naturally occurring soluble form of mouse Toll-like receptor 4 inhibits lipopolysaccharide signaling. J Immunol 2000; 165(12):6682-6686.

Janeway CA, Jr. Approaching the asymptote? Evolution and revolution in immunology. Cold Spring Harb Symp Quant Biol 1989; 54 Pt 1:1-13.

Janeway CA, Jr. The immune system evolved to discriminate infectious nonself from noninfectious self. Immunol Today 1992; 13(1):11-16.

Janssens S, Burns K, Tschopp J, Beyaert R. Regulation of interleukin-1- and lipopolysaccharide-induced NF-kappaB activation by alternative splicing of MyD88. Curr Biol 2002; 12(6):467-471.

Jaresova I, Rozkova D, Spisek R, Janda A, Brazova J, Sediva A. Kinetics of Toll-like receptor-4 splice variants expression in lipopolysaccharide-stimulated antigen presenting cells of healthy donors and patients with cystic fibrosis. Microbes Infect 2007; 9(11):1359-1367.

Jin MS, Kim SE, Heo JY et al. Crystal structure of the TLR1-TLR2 heterodimer induced by binding of a tri-acylated lipopeptide. Cell 2007; 130(6):1071-1082.

Jin MS, Lee JO. Structures of TLR-ligand complexes. Curr Opin Immunol 2008; 20(4):414-419.

Johnson AP, Pearson A, Duckworth G. Surveillance and epidemiology of MRSA bacteraemia in the UK. J Antimicrob Chemother 2005; 56(3):455-462.

Jurk M, Schulte B, Kritzler A et al. C-Class CpG ODN: sequence requirements and characterization of immunostimulatory activities on mRNA level. Immunobiology 2004; 209(1-2):141-154.

Kabesch M, Peters W, Carr D, Leupold W, Weiland SK, von ME. Association between polymorphisms in caspase recruitment domain containing protein 15 and allergy in two German populations. J Allergy Clin Immunol 2003; 111(4):813-817.

Kabesch M, Lauener RP. Why Old McDonald had a farm but no allergies: genes, environments, and the hygiene hypothesis. J Leukoc Biol 2004; 75(3):383-387.

Kahl BC, Goulian M, van WW et al. Staphylococcus aureus RN6390 replicates and induces apoptosis in a pulmonary epithelial cell line. Infect Immun 2000; 68(9):5385-5392.

Kaplowitz LG, Comstock JA, Landwehr DM, Dalton HP, Mayhall CG. Prospective study of microbial colonization of the nose and skin and infection of the vascular access site in hemodialysis patients. J Clin Microbiol 1988; 26(7):1257-1262.

Kasper M, Stosiek P. The expression of vimentin in epithelial cells from human nasal mucosa. Eur Arch Otorhinolaryngol 1990; 248(1):53-56.

Kataoka K, Muta T, Yamazaki S, Takeshige K. Activation of macrophages by linear (1right-arrow3)-beta-D-glucans. Impliations for the recognition of fungi by innate immunity. J Biol Chem 2002; 277(39):36825-36831.

Katoh Y, Shiba Y, Mitsuhashi H, Yanagida Y, Takatsu H, Nakayama K. Tollip and Tom1 form a complex and recruit ubiquitin-conjugated proteins onto early endosomes. J Biol Chem 2004; 279(23):24435-24443.

Katoh Y, Imakagura H, Futatsumori M, Nakayama K. Recruitment of clathrin onto endosomes by the Tom1-Tollip complex. Biochem Biophys Res Commun 2006; 341(1):143-149.

Kessler SW. Rapid isolation of antigens from cells with a staphylococcal protein A-antibody adsorbent: parameters of the interaction of antibody-antigen complexes with protein A. J Immunol 1975; 115(6):1617-1624.

Khor CC, Chapman SJ, Vannberg FO et al. A Mal functional variant is associated with protection against invasive pneumococcal disease, bacteremia, malaria and tuberculosis. Nat Genet 2007; 39(4):523-528.

Kim HM, Park BS, Kim JI et al. Crystal structure of the TLR4-MD-2 complex with bound endotoxin antagonist Eritoran. Cell 2007; 130(5):906-917.

Kim PH, Kagnoff MF. Transforming growth factor beta 1 increases IgA isotype switching at the clonal level. J Immunol 1990; 145(11):3773-3778.

Kimman TG, Banus S, Reijmerink N et al. Association of interacting genes in the toll-like receptor signaling pathway and the antibody response to pertussis vaccination. PLoS One 2008; 3(11):e3665.

Kinjyo I, Hanada T, Inagaki-Ohara K et al. SOCS1/JAB is a negative regulator of LPS-induced macrophage activation. Immunity 2002; 17(5):583-591.

Kirby WM. Extraction of a highly potent penicillin inactivator from penicillin resistant Staphylococci. Science 1944; 99(2579):452-453.

Kluytmans J, van BA, Verbrugh H. Nasal carriage of Staphylococcus aureus: epidemiology, underlying mechanisms, and associated risks. Clin Microbiol Rev 1997; 10(3):505-520.

Kobayashi K, Hernandez LD, Galan JE, Janeway CA, Jr., Medzhitov R, Flavell RA. IRAK-M is a negative regulator of Toll-like receptor signaling. Cell 2002; 110(2):191-202.

Krieg AM, Yi AK, Matson S et al. CpG motifs in bacterial DNA trigger direct B-cell activation. Nature 1995; 374(6522):546-549.

Krieg AM, Wu T, Weeratna R et al. Sequence motifs in adenoviral DNA block immune activation by stimulatory CpG motifs. Proc Natl Acad Sci U S A 1998; 95(21):12631-12636.

Krikos A, Laherty CD, Dixit VM. Transcriptional activation of the tumor necrosis factor alpha-inducible zinc finger protein, A20, is mediated by kappa B elements. J Biol Chem 1992; 267(25):17971-17976.

Kusunoki T, Hailman E, Juan TS, Lichenstein HS, Wright SD. Molecules from Staphylococcus aureus that bind CD14 and stimulate innate immune responses. J Exp Med 1995; 182(6):1673-1682.

Lane AP, Truong-Tran QA, Schleimer RP. Altered expression of genes associated with innate immunity and inflammation in recalcitrant rhinosinusitis with polyps. Am J Rhinol 2006; 20(2):138-144.

LeBouder E, Rey-Nores JE, Rushmere NK et al. Soluble forms of Toll-like receptor (TLR)2 capable of modulating TLR2 signaling are present in human plasma and breast milk. J Immunol 2003; 171(12):6680-6689.

Leemans JC, Vervoordeldonk MJ, Florquin S, van Kessel KP, van der PT. Differential role of interleukin-6 in lung inflammation induced by lipoteichoic acid and peptidoglycan from Staphylococcus aureus. Am J Respir Crit Care Med 2002; 165(10):1445-1450.

Lemaitre B, Nicolas E, Michaut L, Reichhart JM, Hoffmann JA. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. Cell 1996; 86(6):973-983.

Lenert P, Stunz L, Yi AK, Krieg AM, Ashman RF. CpG stimulation of primary mouse B cells is blocked by inhibitory oligodeoxyribonucleotides at a site proximal to NF-kappaB activation. Antisense Nucleic Acid Drug Dev 2001; 11(4):247-256.

Lenert PS. Classification, mechanisms of action, and therapeutic applications of inhibitory oligonucleotides for Toll-like receptors (TLR) 7 and 9. Mediators Inflamm 2010; 2010:986596.

Li T, Hu J, Li L. Characterization of Tollip protein upon Lipopolysaccharide challenge. Mol Immunol 2004; 41(1):85-92.

Li X, Commane M, Burns C, Vithalani K, Cao Z, Stark GR. Mutant cells that do not respond to interleukin-1 (IL-1) reveal a novel role for IL-1 receptor-associated kinase. Mol Cell Biol 1999; 19(7):4643-4652.

Lieber M, Smith B, Szakal A, Nelson-Rees W, Todaro G. A continuous tumor-cell line from a human lung carcinoma with properties of type II alveolar epithelial cells. Int J Cancer 1976; 17(1):62-70.

Lien E, Ingalls RR. Toll-like receptors. Crit Care Med 2002; 30(1 Supp):S1-S11.

Liew FY, Xu D, Brint EK, O'Neill LA. Negative regulation of toll-like receptor-mediated immune responses. Nat Rev Immunol 2005; 5(6):446-458.

Lindbom J, Gustafsson M, Blomqvist G et al. Exposure to wear particles generated from studded tires and pavement induces inflammatory cytokine release from human macrophages. Chem Res Toxicol 2006; 19(4):521-530.

Llarrull LI, Fisher JF, Mobashery S. Molecular basis and phenotype of methicillin resistance in Staphylococcus aureus and insights into new beta-lactams that meet the challenge. Antimicrob Agents Chemother 2009; 53(10):4051-4063.

Lo YL, Beckhouse AG, Boulus SL, Wells CA. Diversification of Tollip isoforms in mouse and man. Mamm Genome 2009; 20(5):305-314.

Lopez-Souza N, Dolganov G, Dubin R et al. Resistance of differentiated human airway epithelium to infection by rhinovirus. Am J Physiol Lung Cell Mol Physiol 2004; 286(2):L373-L381.

Lorenz E, Mira JP, Frees KL, Schwartz DA. Relevance of mutations in the TLR4 receptor in patients with gram-negative septic shock. Arch Intern Med 2002; 162(9):1028-1032.

Lynch JP, III. Hospital-acquired pneumonia: risk factors, microbiology, and treatment. Chest 2001; 119(2 Suppl):373S-384S.

MacRedmond RE, Greene CM, Dorscheid DR, McElvaney NG, O'Neill SJ. Epithelial expression of TLR4 is modulated in COPD and by steroids, salmeterol and cigarette smoke. Respir Res 2007; 8:84.

Maldonado-Baez L, Wendland B. Endocytic adaptors: recruiters, coordinators and regulators. Trends Cell Biol 2006; 16(10):505-513.

Mangili A, Bica I, Snydman DR, Hamer DH. Daptomycin-resistant, methicillin-resistant Staphylococcus aureus bacteremia. Clin Infect Dis 2005; 40(7):1058-1060.

Mansson A, Bachar O, Adner M, Cardell LO. Nasal CpG oligodeoxynucleotide administration induces a local inflammatory response in nonallergic individuals. Allergy 2009; 64(9):1292-1300.

Marty FM, Yeh WW, Wennersten CB et al. Emergence of a clinical daptomycin-resistant Staphylococcus aureus isolate during treatment of methicillin-resistant Staphylococcus aureus bacteremia and osteomyelitis. J Clin Microbiol 2006; 44(2):595-597.

Massova I, Mobashery S. Kinship and diversification of bacterial penicillin-binding proteins and beta-lactamases. Antimicrob Agents Chemother 1998; 42(1):1-17.

Mayer AK, Muehmer M, Mages J et al. Differential recognition of TLR-dependent microbial ligands in human bronchial epithelial cells. J Immunol 2007; 178(5):3134-3142.

McCormick JK, Yarwood JM, Schlievert PM. Toxic shock syndrome and bacterial superantigens: an update. Annu Rev Microbiol 2001; 55:77-104.

McDougall CM, Blaylock MG, Douglas JG, Brooker RJ, Helms PJ, Walsh GM. Nasal epithelial cells as surrogates for bronchial epithelial cells in airway inflammation studies. Am J Respir Cell Mol Biol 2008; 39(5):560-568.

Medzhitov R, Preston-Hurlburt P, Janeway CA, Jr. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature 1997; 388(6640):394-397.

Medzhitov R, Janeway C, Jr. Innate immunity. N Engl J Med 2000; 343(5):338-344.

Medzhitov R. Recognition of microorganisms and activation of the immune response. Nature 2007; 449(7164):819-826.

Medzhitov R. Origin and physiological roles of inflammation. Nature 2008; 454(7203):428-435.

Meka VG, Pillai SK, Sakoulas G et al. Linezolid resistance in sequential Staphylococcus aureus isolates associated with a T2500A mutation in the 23S rRNA gene and loss of a single copy of rRNA. J Infect Dis 2004; 190(2):311-317.

Melmed G, Thomas LS, Lee N et al. Human intestinal epithelial cells are broadly unresponsive to Toll-like receptor 2-dependent bacterial ligands: implications for host-microbial interactions in the gut. J Immunol 2003; 170(3):1406-1415.

Miyake K. Innate immune sensing of pathogens and danger signals by cell surface Toll-like receptors. Semin Immunol 2007; 19(1):3-10.

Mogensen TH, Paludan SR. Reading the viral signature by Toll-like receptors and other pattern recognition receptors. J Mol Med 2005; 83(3):180-192.

Mogensen TH. Pathogen recognition and inflammatory signaling in innate immune defenses. Clin Microbiol Rev 2009; 22(2):240-73, Table.

Moll R, Krepler R, Franke WW. Complex cytokeratin polypeptide patterns observed in certain human carcinomas. Differentiation 1983; 23(3):256-269.

Moore CE, Sandberg A, . Studies of a human tumor cell line with a diploid karyotype. Cancer 1964; 17:170-175.

Moore CE, Segal S, Berendt AR, Hill AV, Day NP. Lack of association between Toll-like receptor 2 polymorphisms and susceptibility to severe disease caused by Staphylococcus aureus. Clin Diagn Lab Immunol 2004; 11(6):1194-1197.

Moorhead PS. Human tumor cell line with a quasi-diploid karyotype (RPMI 2650). Exp Cell Res 1965; 39(1):190-196.

Morgan AJ, Parker S. Translational mini-review series on vaccines: The Edward Jenner Museum and the history of vaccination. Clin Exp Immunol 2007; 147(3):389-394.

Moyron-Quiroz JE, Rangel-Moreno J, Kusser K et al. Role of inducible bronchus associated lymphoid tissue (iBALT) in respiratory immunity. Nat Med 2004; 10(9):927-934.

Murakami H, Masui H. Hormonal control of human colon carcinoma cell growth in serum-free medium. Proc Natl Acad Sci U S A 1980; 77(6):3464-3468.

Myhre AE, Stuestol JF, Dahle MK et al. Organ injury and cytokine release caused by peptidoglycan are dependent on the structural integrity of the glycan chain. Infect Immun 2004; 72(3):1311-1317.

Nakagawa R, Naka T, Tsutsui H et al. SOCS-1 participates in negative regulation of LPS responses. Immunity 2002; 17(5):677-687.

Nakao Y, Funami K, Kikkawa S et al. Surface-expressed TLR6 participates in the recognition of diacylated lipopeptide and peptidoglycan in human cells. J Immunol 2005; 174(3):1566-1573.

Neumann D, Kollewe C, Resch K, Martin MU. The death domain of IRAK-1: an oligomerization domain mediating interactions with MyD88, Tollip, IRAK-1, and IRAK-4. Biochem Biophys Res Commun 2007; 354(4):1089-1094.

Nystrom K, Biller M, Grahn A, Lindh M, Larson G, Olofsson S. Real time PCR for monitoring regulation of host gene expression in herpes simplex virus type 1-infected human diploid cells. J Virol Methods 2004; 118(2):83-94.

O'Brien GJ, Riddell G, Elborn JS, Ennis M, Skibinski G. Staphylococcus aureus enterotoxins induce IL-8 secretion by human nasal epithelial cells. Respir Res 2006; 7:115.

O'Brien LM, Walsh EJ, Massey RC, Peacock SJ, Foster TJ. Staphylococcus aureus clumping factor B (ClfB) promotes adherence to human type I cytokeratin 10: implications for nasal colonization. Cell Microbiol 2002; 4(11):759-770.

O'Riordan K, Lee JC. Staphylococcus aureus capsular polysaccharides. Clin Microbiol Rev 2004; 17(1):218-234.

Ohnuma K, Yamochi T, Uchiyama M et al. CD26 mediates dissociation of Tollip and IRAK-1 from caveolin-1 and induces upregulation of CD86 on antigen-presenting cells. Mol Cell Biol 2005; 25(17):7743-7757.

Ohto U, Fukase K, Miyake K, Satow Y. Crystal structures of human MD-2 and its complex with antiendotoxic lipid IVa. Science 2007; 316(5831):1632-1634.

Okamura H, Tsutsi H, Komatsu T et al. Cloning of a new cytokine that induces IFN-gamma production by T cells. Nature 1995; 378(6552):88-91.

Opipari AW, Jr., Boguski MS, Dixit VM. The A20 cDNA induced by tumor necrosis factor alpha encodes a novel type of zinc finger protein. J Biol Chem 1990; 265(25):14705-14708.

Otte JM, Cario E, Podolsky DK. Mechanisms of cross hyporesponsiveness to Toll-like receptor bacterial ligands in intestinal epithelial cells. Gastroenterology 2004; 126(4):1054-1070.

Otte JM, Podolsky DK. Functional modulation of enterocytes by gram-positive and gram-negative microorganisms. Am J Physiol Gastrointest Liver Physiol 2004; 286(4):G613-G626.

Ozinsky A, Underhill DM, Fontenot JD et al. The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors. Proc Natl Acad Sci U S A 2000; 97(25):13766-13771.

Pan C, Kumar C, Bohl S, Klingmueller U, Mann M. Comparative proteomic phenotyping of cell lines and primary cells to assess preservation of cell type-specific functions. Mol Cell Proteomics 2009; 8(3):443-450.

Parker LC, Whyte MK, Vogel SN, Dower SK, Sabroe I. Toll-like receptor (TLR)2 and TLR4 agonists regulate CCR expression in human monocytic cells. J Immunol 2004; 172(8):4977-4986.

Parnaby RM, O'Dwyer G, Monsey HA, Shafi MS. Carriage of Staphylococcus aureus in the elderly. J Hosp Infect 1996; 33(3):201-206.

Paul TR, Venter A, Blaszczak LC, Parr TR, Jr., Labischinski H, Beveridge TJ. Localization of penicillin-binding proteins to the splitting system of Staphylococcus aureus septa by using a mercury-penicillin V derivative. J Bacteriol 1995; 177(13):3631-3640.

Peacock SJ, Justice A, Griffiths D et al. Determinants of acquisition and carriage of Staphylococcus aureus in infancy. J Clin Microbiol 2003; 41(12):5718-5725.

Pelaia G, Cuda G, Vatrella A et al. Effects of transforming growth factor-[beta] and budesonide on mitogen-activated protein kinase activation and apoptosis in airway epithelial cells. Am J Respir Cell Mol Biol 2003; 29(1):12-18.

Piao W, Song C, Chen H et al. Endotoxin tolerance dysregulates MyD88- and Toll/IL-1R domain-containing adapter inducing IFN-beta-dependent pathways and increases expression of negative regulators of TLR signaling. J Leukoc Biol 2009; 86(4):863-875.

Platz J, Beisswenger C, Dalpke A et al. Microbial DNA induces a host defense reaction of human respiratory epithelial cells. J Immunol 2004; 173(2):1219-1223.

Poltorak A, He X, Smirnova I et al. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science 1998; 282(5396):2085-2088.

Pujol M, Pena C, Pallares R et al. Nosocomial Staphylococcus aureus bacteremia among nasal carriers of methicillin-resistant and methicillin-susceptible strains. Am J Med 1996; 100(5):509-516.

Ramanathan M, Jr., Lane AP. A comparison of experimental methods in molecular chronic rhinosinusitis research. Am J Rhinol 2007; 21(3):373-377.

Ramanathan M, Jr., Lee WK, Spannhake EW, Lane AP. Th2 cytokines associated with chronic rhinosinusitis with polyps down-regulate the antimicrobial immune function of human sinonasal epithelial cells. Am J Rhinol 2008; 22(2):115-121.

Rello J, Torres A, Ricart M et al. Ventilator-associated pneumonia by Staphylococcus aureus. Comparison of methicillin-resistant and methicillin-sensitive episodes. Am J Respir Crit Care Med 1994; 150(6 Pt 1):1545-1549.

Ritz HL, Kirkland JJ, Bond GG, Warner EK, Petty GP. Association of high levels of serum antibody to staphylococcal toxic shock antigen with nasal carriage of toxic shock antigen-producing strains of Staphylococcus aureus. Infect Immun 1984; 43(3):954-958.

Robinson DA, Kearns AM, Holmes A et al. Re-emergence of early pandemic Staphylococcus aureus as a community-acquired meticillin-resistant clone. Lancet 2005; 365(9466):1256-1258.

Roche FM, Meehan M, Foster TJ. The Staphylococcus aureus surface protein SasG and its homologues promote bacterial adherence to human desquamated nasal epithelial cells. Microbiology 2003; 149(Pt 10):2759-2767.

Roger T, Bresser P, Snoek M et al. Exaggerated IL-8 and IL-6 responses to TNF-alpha by parainfluenza virus type 4-infected NCI-H292 cells. Am J Physiol Lung Cell Mol Physiol 2004; 287(5):L1048-L1055.

Saitoh S, Akashi S, Yamada T et al. Lipid A antagonist, lipid IVa, is distinct from lipid A in interaction with Toll-like receptor 4 (TLR4)-MD-2 and ligand-induced TLR4 oligomerization. Int Immunol 2004; 16(7):961-969.

Salib RJ, Lau LC, Howarth PH. The novel use of the human nasal epithelial cell line RPMI 2650 as an in vitro model to study the influence of allergens and cytokines on transforming growth factor-beta gene expression and protein release. Clin Exp Allergy 2005; 35(6):811-819.

Schimming TT, Parwez Q, Petrasch-Parwez E, Nothnagel M, Epplen JT, Hoffjan S. Association of toll-interacting protein gene polymorphisms with atopic dermatitis. BMC Dermatol 2007; 7:3.

Schmidt M, Hagen P, Lang S, Ditzinger G, Merkle H. In vitro cell models to study nasal mucosal permeability and metabolism. Advanced drug delivery reviews 1998; 29:51-79.

Schumann RR, Leong SR, Flaggs GW et al. Structure and function of lipopolysaccharide binding protein. Science 1990; 249(4975):1429-1431.

Schwandner R, Dziarski R, Wesche H, Rothe M, Kirschning CJ. Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by toll-like receptor 2. J Biol Chem 1999; 274(25):17406-17409.

Schwartz DA, Quinn TJ, Thorne PS, Sayeed S, Yi AK, Krieg AM. CpG motifs in bacterial DNA cause inflammation in the lower respiratory tract. J Clin Invest 1997; 100(1):68-73.

Shibolet O, Podolsky DK. TLRs in the Gut. IV. Negative regulation of Toll-like receptors and intestinal homeostasis: addition by subtraction. Am J Physiol Gastrointest Liver Physiol 2007; 292(6):G1469-G1473.

Shinefield HR, Ribble J, Boris M, Eichenwald H. Bacterial interference: its effect on nursery-acquired infection with Staphylococcus aureus. I. Preliminary observations on artificial colonzation of newborns. Am J Dis Child 1963; 105:646-654.

Shull MM, Ormsby I, Kier AB et al. Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease. Nature 1992; 359(6397):693-699.

Sievert DM, Rudrik JT, Patel JB, McDonald LC, Wilkins MJ, Hageman JC. Vancomycin-resistant Staphylococcus aureus in the United States, 2002-2006. Clin Infect Dis 2008; 46(5):668-674.

Sivori S, Carlomagno S, Moretta L, Moretta A. Comparison of different CpG oligodeoxynucleotide classes for their capability to stimulate human NK cells. Eur J Immunol 2006; 36(4):961-967.

Skiest DJ. Treatment failure resulting from resistance of Staphylococcus aureus to daptomycin. J Clin Microbiol 2006; 44(2):655-656.

Stacey KJ, Young GR, Clark F et al. The molecular basis for the lack of immunostimulatory activity of vertebrate DNA. J Immunol 2003; 170(7):3614-3620.

Standards Unit EaSLCfIHPA. National Standard Method of the identification of Staphylococcus species, micrococcus species and rothia species. [2.1], 1-17. 2007. Ref Type: Generic

Standiford TJ, Arenberg DA, Danforth JM, Kunkel SL, VanOtteren GM, Strieter RM. Lipoteichoic acid induces secretion of interleukin-8 from human blood monocytes: a cellular and molecular analysis. Infect Immun 1994; 62(1):119-125.

Stewart G, Holt R. Evolutio of natural resistance to the newer penicillins. Br Med J 1963; 1(5326):308-311.

Strober W, Murray PJ, Kitani A, Watanabe T. Signalling pathways and molecular interactions of NOD1 and NOD2. Nat Rev Immunol 2006; 6(1):9-20.

Stumbles PA, Thomas JA, Pimm CL et al. Resting respiratory tract dendritic cells preferentially stimulate T helper cell type 2 (Th2) responses and require obligatory cytokine signals for induction of Th1 immunity. J Exp Med 1998; 188(11):2019-2031.

Sugai M, Yamada S, Nakashima S et al. Localized perforation of the cell wall by a major autolysin: atl gene products and the onset of penicillin-induced lysis of Staphylococcus aureus. J Bacteriol 1997; 179(9):2958-2962.

Sugawara I, Yamada H, Li C, Mizuno S, Takeuchi O, Akira S. Mycobacterial infection in TLR2 and TLR6 knockout mice. Microbiol Immunol 2003; 47(5):327-336.

Suzuki N, Suzuki S, Duncan GS et al. Severe impairment of interleukin-1 and Toll-like receptor signalling in mice lacking IRAK-4. Nature 2002; 416(6882):750-756.

Takeuchi O, Hoshino K, Kawai T et al. Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. Immunity 1999; 11(4):443-451.

Takeuchi O, Kawai T, Sanjo H et al. TLR6: A novel member of an expanding toll-like receptor family. Gene 1999; 231(1-2):59-65.

Takeuchi O, Hoshino K, Akira S. Cutting edge: TLR2-deficient and MyD88-deficient mice are highly susceptible to Staphylococcus aureus infection. J Immunol 2000; 165(10):5392-5396.

Takeuchi O, Kaufmann A, Grote K et al. Cutting edge: preferentially the R-stereoisomer of the mycoplasmal lipopeptide macrophage-activating lipopeptide-2 activates immune cells through a toll-like receptor 2- and MyD88-dependent signaling pathway. J Immunol 2000; 164(2):554-557.

Takeuchi O, Sato S, Horiuchi T et al. Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins. J Immunol 2002; 169(1):10-14.

Tao K, Fujii M, Tsukumo S et al. Genetic variations of Toll-like receptor 9 predispose to systemic lupus erythematosus in Japanese population. Ann Rheum Dis 2007; 66(7):905-909.

Tedgui A, Mallat Z. Cytokines in atherosclerosis: pathogenic and regulatory pathways. Physiol Rev 2006; 86(2):515-581.

Teleshova N, Kenney J, Van NG et al. Local and systemic effects of intranodally injected CpG-C immunostimulatory-oligodeoxyribonucleotides in macaques. J Immunol 2006; 177(12):8531-8541.

Timmerman CP, Mattsson E, Martinez-Martinez L et al. Induction of release of tumor necrosis factor from human monocytes by staphylococci and staphylococcal peptidoglycans. Infect Immun 1993; 61(10):4167-4172.

Togbe D, Schnyder-Candrian S, Schnyder B et al. TLR4 gene dosage contributes to endotoxin-induced acute respiratory inflammation. J Leukoc Biol 2006; 80(3):451-457.

Tokunaga T, Yamamoto H, Shimada S et al. Antitumor activity of deoxyribonucleic acid fraction from Mycobacterium bovis BCG. I. Isolation, physicochemical characterization, and antitumor activity. J Natl Cancer Inst 1984; 72(4):955-962.

Tokunaga T, Yano O, Kuramoto E et al. Synthetic oligonucleotides with particular base sequences from the cDNA encoding proteins of Mycobacterium bovis BCG induce interferons and activate natural killer cells. Microbiol Immunol 1992; 36(1):55-66.

Travassos LH, Girardin SE, Philpott DJ et al. Toll-like receptor 2-dependent bacterial sensing does not occur via peptidoglycan recognition. EMBO Rep 2004; 5(10):1000-1006.

Travis SM, Conway BA, Zabner J et al. Activity of abundant antimicrobials of the human airway. Am J Respir Cell Mol Biol 1999; 20(5):872-879.

Triantafilou M, Gamper FG, Haston RM et al. Membrane sorting of toll-like receptor (TLR)-2/6 and TLR2/1 heterodimers at the cell surface determines heterotypic associations with CD36 and intracellular targeting. J Biol Chem 2006; 281(41):31002-31011.

Trieu A, Bokil N, Dunn JA et al. TLR9-independent effects of inhibitory oligonucleotides on macrophage responses to S. typhimurium. Immunol Cell Biol 2009; 87(3):218-225.

Tsiodras S, Gold HS, Sakoulas G et al. Linezolid resistance in a clinical isolate of Staphylococcus aureus. Lancet 2001; 358(9277):207-208.

Uehara Y, Nakama H, Agematsu K et al. Bacterial interference among nasal inhabitants: eradication of Staphylococcus aureus from nasal cavities by artificial implantation of Corynebacterium sp. J Hosp Infect 2000; 44(2):127-133.

Unanue ER. Studies in listeriosis show the strong symbiosis between the innate cellular system and the T-cell response. Immunol Rev 1997; 158:11-25.

Underhill DM, Ozinsky A, Hajjar AM et al. The Toll-like receptor 2 is recruited to macrophage phagosomes and discriminates between pathogens. Nature 1999; 401(6755):811-815.

van BA. Staphylococcal colonization and infection: homeostasis versus disbalance of human (innate) immunity and bacterial virulence. Curr Opin Infect Dis 2006; 19(4):339-344.

van HJ. Formation of the glycan chains in the synthesis of bacterial peptidoglycan. Glycobiology 2001; 11(3):25R-36R.

Vandenesch F, Naimi T, Enright MC et al. Community-acquired methicillin-resistant Staphylococcus aureus carrying Panton-Valentine leukocidin genes: worldwide emergence. Emerg Infect Dis 2003; 9(8):978-984.

Vandermeer J, Sha Q, Lane AP, Schleimer RP. Innate immunity of the sinonasal cavity: expression of messenger RNA for complement cascade components and toll-like receptors. Arch Otolaryngol Head Neck Surg 2004; 130(12):1374-1380.

Vandesompele J, De PA, Speleman F. Elimination of primer-dimer artifacts and genomic coamplification using a two-step SYBR green I real-time RT-PCR. Anal Biochem 2002; 303(1):95-98.

Verkaik NJ, Lebon A, de Vogel CP et al. Induction of antibodies by Staphylococcus aureus nasal colonization in young children. Clin Microbiol Infect 2010; 16(8):1312-1317.

Vikram HR, Havill NL, Koeth LM, Boyce JM. Clinical progression of methicillin-resistant Staphylococcus aureus vertebral osteomyelitis associated with reduced susceptibility to daptomycin. J Clin Microbiol 2005; 43(10):5384-5387.

Vollmer J, Weeratna R, Payette P et al. Characterization of three CpG oligodeoxynucleotide classes with distinct immunostimulatory activities. Eur J Immunol 2004; 34(1):251-262.

Vollmer W, Blanot D, de Pedro MA. Peptidoglycan structure and architecture. FEMS Microbiol Rev 2008; 32(2):149-167.

von AS, Morath S, Hareng L et al. Lipoteichoic acid from Staphylococcus aureus is a potent stimulus for neutrophil recruitment. Immunobiology 2003; 208(4):413-422.

von AS, Schroder NW, Traub S et al. Heterozygous toll-like receptor 2 polymorphism does not affect lipoteichoic acid-induced chemokine and inflammatory responses. Infect Immun 2004; 72(3):1828-1831.

von AS, Deininger S, Draing C, Gueinzius K, Dehus O, Hermann C. Gender difference in cytokine secretion on immune stimulation with LPS and LTA. J Interferon Cytokine Res 2006; 26(12):887-892.

von AS, Hartung T, Hermann C. Comment on "Not lipoteichoic acid but lipoproteins appear to be the dominant immunobiologically active compounds in Staphylococcus aureus". J Immunol 2007; 178(5):2610-2611.

von EC, Becker K, Machka K, Stammer H, Peters G. Nasal carriage as a source of Staphylococcus aureus bacteremia. Study Group. N Engl J Med 2001; 344(1):11-16.

Walsh EJ, Miajlovic H, Gorkun OV, Foster TJ. Identification of the Staphylococcus aureus MSCRAMM clumping factor B (ClfB) binding site in the alphaC-domain of human fibrinogen. Microbiology 2008; 154(Pt 2):550-558.

Wang C, Deng L, Hong M, Akkaraju GR, Inoue J, Chen ZJ. TAK1 is a ubiquitin-dependent kinase of MKK and IKK. Nature 2001; 412(6844):346-351.

Wang.J., Matsukura S, Watanabe S, Adachi M, Suzaki H. Involvement of Toll-like receptors in the immune response of nasal polyp epithelial cells. Clinical Immunology 2007; 124:345-352.

Washington WAS, Janda W, Koneman E, Procop G, Schreckenberger P, Woods G. Medical bacteriology: Taxonomy, Morphology, Physiology and Virulence. In: Koneman's, editor. Color Atlas and Textbook of Diagnostic Microbiology. Lippincott Williams & Wilkins, 2006: 167-211.

Watanabe H, Masaki H, Asoh N et al. Molecular analysis of methicillin-resistant Staphylococcus aureus as a causative agent of bronchopulmonary infection: relation to colonization in the upper respiratory tract. J Clin Microbiol 2000; 38(10):3867-3869.

Watanabe T, Kitani A, Murray PJ, Strober W. NOD2 is a negative regulator of Toll-like receptor 2-mediated T helper type 1 responses. Nat Immunol 2004; 5(8):800-808.

Watanabe T, Kitani A, Strober W. NOD2 regulation of Toll-like receptor responses and the pathogenesis of Crohn's disease. Gut 2005; 54(11):1515-1518.

Weiner HL, Friedman A, Miller A et al. Oral tolerance: immunologic mechanisms and treatment of animal and human organ-specific autoimmune diseases by oral administration of autoantigens. Annu Rev Immunol 1994; 12:809-837.

Weinke T, Schiller R, Fehrenbach FJ, Pohle HD. Association between Staphylococcus aureus nasopharyngeal colonization and septicemia in patients infected with the human immunodeficiency virus. Eur J Clin Microbiol Infect Dis 1992; 11(11):985-989.

Wells CA, Chalk AM, Forrest A et al. Alternate transcription of the Toll-like receptor signaling cascade. Genome Biol 2006; 7(2):R10.

Wertheim HF, Vos MC, Ott A et al. Risk and outcome of nosocomial Staphylococcus aureus bacteraemia in nasal carriers versus non-carriers. Lancet 2004; 364(9435):703-705.

Wertheim HF, Melles DC, Vos MC et al. The role of nasal carriage in Staphylococcus aureus infections. Lancet Infect Dis 2005; 5(12):751-762.

Wertheim HF, Walsh E, Choudhurry R et al. Key role for clumping factor B in Staphylococcus aureus nasal colonization of humans. PLoS Med 2008; 5(1):e17.

Wesche H, Gao X, Li X, Kirschning CJ, Stark GR, Cao Z. IRAK-M is a novel member of the Pelle/interleukin-1 receptor-associated kinase (IRAK) family. J Biol Chem 1999; 274(27):19403-19410.

West AP, Koblansky AA, Ghosh S. Recognition and signaling by toll-like receptors. Annu Rev Cell Dev Biol 2006; 22:409-437.

Williams JV, Vowels B, Honig P, Leyden JJ. Staphylococcus aureus isolation from the lesions, the hands, and anterior nares of patients with atopic dermatitis. J Emerg Med 1999; 17(1):207-211.

Williams R. Healthy carriage of Staphylococcus aureus: its prevalence and importance. Bacteriol Rev 1963; 27:56-71.

Wistuba II, Bryant D, Behrens C et al. Comparison of features of human lung cancer cell lines and their corresponding tumors. Clin Cancer Res 1999; 5(5):991-1000.

Witherden IR, Vanden Bon EJ, Goldstraw P, Ratcliffe C, Pastorino U, Tetley TD. Primary human alveolar type II epithelial cell chemokine release: effects of cigarette smoke and neutrophil elastase. Am J Respir Cell Mol Biol 2004; 30(4):500-509.

Woodford N, Livermore DM. Infections caused by Gram-positive bacteria: a review of the global challenge. J Infect 2009; 59 Suppl 1:S4-16.

Wright SD, Ramos RA, Tobias PS, Ulevitch RJ, Mathison JC. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. Science 1990; 249(4975):1431-1433.

Wyllie DH, Kiss-Toth E, Visintin A et al. Evidence for an accessory protein function for Toll-like receptor 1 in anti-bacterial responses. J Immunol 2000; 165(12):7125-7132.

Xiang M, Fan J. Pattern recognition receptor-dependent mechanisms of acute lung injury. Mol Med 2010; 16(1-2):69-82.

Yamada H, Gursel I, Takeshita F et al. Effect of suppressive DNA on CpG-induced immune activation. J Immunol 2002; 169(10):5590-5594.

Yamakami M, Yoshimori T, Yokosawa H. Tom1, a VHS domain-containing protein, interacts with tollip, ubiquitin, and clathrin. J Biol Chem 2003; 278(52):52865-52872.

Yamamoto M, Sato S, Hemmi H et al. Essential role for TIRAP in activation of the signalling cascade shared by TLR2 and TLR4. Nature 2002; 420(6913):324-329.

Yamamoto M, Sato S, Hemmi H et al. TRAM is specifically involved in the Toll-like receptor 4-mediated MyD88-independent signaling pathway. Nat Immunol 2003; 4(11):1144-1150.

Yamamoto M, Sato S, Hemmi H et al. Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. Science 2003; 301(5633):640-643.

Yeo SJ, Yoon JG, Hong SC, Yi AK. CpG DNA induces self and cross-hyporesponsiveness of RAW264.7 cells in response to CpG DNA and lipopolysaccharide: alterations in IL-1 receptor-associated kinase expression. J Immunol 2003; 170(2):1052-1061.

Yoshida H, Jono H, Kai H, Li JD. The tumor suppressor cylindromatosis (CYLD) acts as a negative regulator for toll-like receptor 2 signaling via negative cross-talk with TRAF6 AND TRAF7. J Biol Chem 2005; 280(49):41111-41121.

Yoshimura A, Takada H, Kaneko T, Kato I, Golenbock D, Hara Y. Structural requirements of muramylpeptides for induction of Toll-like receptor 2-mediated NF-kappaB activation in CHO cells. J Endotoxin Res 2000; 6(5):407-410.

Zahringer U, Lindner B, Inamura S, Heine H, Alexander C. TLR2 - promiscuous or specific? A critical re-evalulation of a receptor expressing apparent broad specificity. Immunobiology 2008; 213(3-4):205-224.

Zarember KA, Godowski PJ. Tissue expression of human Toll-like receptors and differential regulation of Toll-like receptor mRNAs in leukocytes in response to microbes, their products, and cytokines. J Immunol 2002; 168(2):554-561.

Zetola N, Francis JS, Nuermberger EL, Bishai WR. Community-acquired meticillin-resistant Staphylococcus aureus: an emerging threat. Lancet Infect Dis 2005; 5(5):275-286.

Zhang G, Ghosh S. Negative regulation of toll-like receptor-mediated signaling by Tollip. J Biol Chem 2002; 277(9):7059-7065.

Zhang Z, Louboutin JP, Weiner DJ, Goldberg JB, Wilson JM. Human airway epithelial cells sense Pseudomonas aeruginosa infection via recognition of flagellin by Toll-like receptor 5. Infect Immun 2005; 73(11):7151-7160.

Zhao J. Sumoylation regulates diverse biological processes. Cell Mol Life Sci 2007; 64(23):3017-3033.

Zhong H, Simons JW. Direct comparison of GAPDH, beta-actin, cyclophilin, and 28S rRNA as internal standards for quantifying RNA levels under hypoxia. Biochem Biophys Res Commun 1999; 259(3):523-526.